Binding of Hydrophobic D-Galactopyranosides to the Lactose Permease of *Escherichia coli*†

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Received April 24, 2002; Revised Manuscript Received August 12, 2002

ABSTRACT: Binding of α- and β-D-galactopyranosides with different hydrophobic aglycons was compared using substrate protection against *N*-ethylmaleimide alkylation of single-Cys148 lactose permease. As demonstrated previously, methyl- or allyl-substituted α-D-galactopyranosides exhibit a 60-fold increase in binding affinity ($K_D = 0.5$ mM), relative to galactose ($K_D = 30$ mM), while methyl β-D-galactopyranoside binds only 3-fold better. In the present study, galactopyranosides with cyclohexyl or phenyl substitutions, both in α and β anomeric configurations, were synthesized. Surprisingly, relative to methyl α-D-galactopyranoside, binding of cyclohexyl α-D-galactopyranoside to lactose permease is essentially unchanged ($K_D = 0.4$ mM), and phenyl α-D-galactopyranoside exhibits only a modest increase in binding affinity ($K_D = 0.15$ mM). Nitro- or methyl-substituted phenyl α-D-galactopyranosides bind with significantly higher affinities ($K_D = 0.014-0.067$ mM), and the strongest binding is observed with analogues containing para substituents. In contrast, D-galactopyranosides with a variety of large hydrophobic substituents (isopropyl, cyclohexyl, phenyl, *o*- or *p*-nitrophenyl) in β anomeric configuration exhibit uniformly weak binding ($K_D = 1.0-2.3$ mM). The results confirm and extend previous observations that hydrophobic aglycons of D-galactopyranosides increase binding affinity, with a clear predilection toward α-substituted sugars. In addition, the data suggest that the primary interaction between the permease and hydrophobic aglycons is directed toward the carbon atom bonded to the anomeric oxygen. The different positioning of this carbon atom in α- or β-D-galactopyranosides thus may provide a rationale for the characteristic binding preference of the permease for α anomers.

The lactose permease (lac permease)[1] of *Escherichia coli* catalyzes the coupled stoichiometric translocation of lactose or other D-galactopyranosides and H^+. This polytopic membrane protein contains 12 hydrophobic, membrane-spanning helices and is a representative of secondary active transport proteins that transduce free energy stored in electrochemical ion gradients into work in the form of a concentration gradient. 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. In addition, experimental observations from structural and extensive mutational analysis have led to a proposed mechanism for lactose/H^+ symport (reviewed in ref *1*).

Ligand binding to the permease requires an α- or β-galactopyranosyl ring of the D-series, and D-galactose is the smallest specific transport substrate, while glucose or glucosides are not recognized or transported (*2*−*6*). A recent study examined the binding of 31 structural analogues of D-galactose to single-Cys148 permease by site-directed *N*-[1-^{14}C]ethylmaleimide ([^{14}C]NEM) labeling of Cys148 which is protected by substrate (*5*). D-Galactose blocks alkylation of Cys148 with a 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 (D-talose) binds almost as well as D-galactose. No substrate protection is observed with deoxy derivatives at C-2, C-3, C-4, or C-6 positions. Due to the very low affinity of D-galactose, the reference compound in these 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. In a more recent study, a series of deoxy and methoxy analogues of the high-affinity ligand *p*-nitrophenyl α-D-galactopyranoside was used to address this question (*6*). *p*-Nitrophenyl α-D-galactopyranoside blocks NEM-alkylation of Cys148 with an apparent affinity of approximately 0.014 mM. A deoxy derivative at position C-2 binds with 25-fold lower affinity, the deoxy analogue at C-3 exhibits ca. 70-fold decreased binding, while binding of the 6-deoxy sugar is at least 130-fold diminished. Remarkably, the C-4 deoxy derivative binds with almost 1500-fold reduced affinity. No

† This work was supported in part by NIH Grant DK51131:06 to H.R.K.

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[1] Abbreviations: lac permease, lactose permease; NEM, *N*-ethylmaleimide; DDM, dodecyl β-D-maltopyranoside; KP_i, potassium phosphate; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; RSO, right-side-out.

10.1021/bi0203076 CCC: $22.00
Published on Web 10/04/2002

significant substrate protection is afforded by analogues with methoxy (CH_3O-) substitutions, with the exception of the C-2 methoxy analogue that binds almost normally. Taken together, the findings indicate that binding of D-galactopyranosides to the permease is governed by H-bond interactions at C-2, C-3, C-4, and C-6 OH groups. Furthermore, the C-4 hydroxyl is the major determinant for ligand binding, suggesting that sugar recognition in lactose permease may have evolved to discriminate primarily between gluco- and galactopyranosides.

One notable observation from these studies is the dramatic difference between the affinities of methyl α- and β-D-galactopyranosides. Methyl or allyl α-D-galactopyanosides exhibit a 60-fold increase in binding affinity ($K_D = 0.5$ mM) relative to D-galactose, while methyl β-D-galactopyranoside binds only 3 times better than galactose (*5*). Thus, while both anomers bind better than D-galactose, methyl α-D-galactopyranoside has a 20-fold greater affinity than β. In the present study, a systematic investigation was undertaken to identify the physicochemical features of hydrophobic aglycons that determine binding affinity to the permease.

EXPERIMENTAL PROCEDURES

Materials. N-([1-^{14}C]Ethyl)maleimide (40 mCi/mmol, in pentane) was purchased from DuPont NEN (Boston, MA). Water (0.5 mL) was added, and the pentane phase was evaporated under argon. The aqueous [^{14}C]NEM stock solution (2.5 mM concentration) was stored at −80 °C. Immobilized monomeric avidin (ImmunoPure) was from Pierce (Rockford, IL). Unless stated otherwise, sugars were obtained from Sigma (St. Louis, MO). Reaction solvent CH_2Cl_2 was distilled from CaH_2. Anhydrous MeOH was purchased from Aldrich (St. Louis, MO). Other solvents and reagents used for the synthesis of galactopyranosides were ACS-grade. Flash column chromatography (*7*) was performed on 32−63 μm silica gel, purchased from Bodman (Aston, PA).

General Methods. ^{1}H NMR spectra were recorded on a Bruker instrument at either 400 or 500 MHz and ^{13}C NMR spectra were recorded at either 100 or 125 MHz, as reference to residual signals of $CDCl_3$ (δ 7.26 for ^{1}H, δ 77.0 for ^{13}C), MeOH-d_4 (δ 49.0 for ^{13}C), and D_2O (δ 4.79 for ^{1}H). Chemical shifts are given in parts per million (δ). Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broadened). Coupling constants, *J*, are reported in Hertz (Hz). TLC was performed on Bodman silica plates (precoated on plastic sheets, 0.20 mm thickness). Spots were visualized with orcinol and/or vanillin stain.

Synthesis of Cyclohexyl, Phenyl, and 4-Methylphenyl D-Galactopyranosides. These galactopyranosides were prepared by $SnCl_4$-catalyzed glycosylation (*8*) of penta-*O*-acetyl-β-D-galactopyranose (*9*) with the corresponding alcohol, followed by Zemplén de-*O*-acetylation.

Synthesis of Cyclohexyl Tetra-O-*acetyl*-α/β-D-*galactopyranosides.* A 1 M solution of $SnCl_4$ in dry CH_2Cl_2 (2.56 mL, 2.56 mmol) was added to a stirred solution of penta-*O*-acetyl-β-D-galactopyranose (1.0 g, 2.56 mmol) in dry CH_2Cl_2 (20 mL) at room temperature under argon. After 10 min, the reaction mixture was cooled to 0 °C in an ice bath, and cyclohexanol (0.6 mL, 5.62 mmol) was added. The solution was stirred overnight at room temperature, washed with ice-cold saturated $NaHCO_3$, and extracted with $CHCl_3$. The combined organic layers were washed with water, dried over anhydrous $MgSO_4$, and concentrated to a yellow oil. The residue was purified by flash column chromatography with hexanes−EtOAc (2:1, v/v). The first fraction yielded the α anomer (*10*) (0.38 g, 35%) as a colorless solid: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.45 (br d, $J_{4,3} = 3.0$ Hz, 1H, H-4), 5.35 (dd, $J_{2,3} = 10.8$ Hz, $J_{2,1} = 3.5$ Hz, 1H, H-2), 5.25 (d, $J_{1,2} = 3.5$ Hz, 1H, H-1), 5.05 (dd, $J_{3,2} = 10.8$ Hz, $J_{3,4} = 3.7$ Hz, 1H, H-3), 4.32 (br t, $J_{5,6a} = J_{5,6b} = 6.5$ Hz, 1H, H-5), 4.06 (dd, $J_{6a,6b} = 11.2$ Hz, $J_{6a,5} = 6.4$ Hz, 1H, H-6a), 4.11 (dd, $J_{6b,6a} = 11.2$ Hz, $J_{6b,5} = 7.0$ Hz, 1H, H-6b), 3.54 (m, 1H, cyclohexyl H-1), 2.13 (s, 3H), 2.06 (s, 3H), 2.03 (s, 3H), and 1.99 (s, 3H) (4xOAc),1.89−1.26 (m, 10H, cyclohexyl 5 x C*H*$_2$).

The second fraction yielded the β anomer (*10*) (0.07 g, 6.4%) as a colorless solid: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.38 (br d, $J_{4,3} = 3.0$ Hz, 1H, H-4), 5.19 (dd, $J_{2,3} = 10.5$ Hz, $J_{2,1} = 7.9$ Hz, 1H, H-2), 5.01 (dd, $J_{3,2} = 10.5$ Hz, $J_{3,4} = 3.4$ Hz, 1H, H-3), 4.54 (d, $J_{1,2} = 7.9$ Hz, 1H, H-1), 4.20 (dd, $J_{6a,6b} = 11.2$ Hz, $J_{6a,5} = 6.4$ Hz, 1H, H-6a), 4.09 (dd, $J_{6b,6a} = 11.1$ Hz, $J_{6b,5} = 7.2$ Hz, 1H, H-6b), 3.88 (br t, $J_{5,6a} = J_{5,6b} = 6.5$ Hz, 1H, H-5), 3.60 (m, 1H, cyclohexyl H-1), 2.14 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H), and 1.98 (s, 3H) (4xOAc), 1.88−1.27 (m, 10H, cyclohexyl, 5 × C*H*$_2$).

Synthesis of Phenyl Tetra-O-acetyl-α/β-D-*galactopyranosides.* Glycosylation of penta-*O*-acetyl-β-D-galactopyranose with phenol was carried out in a similar manner. The products were purified by flash column chromatography with hexanes−EtOAc (2:1, v/v). The first fraction yielded the α anomer (*11*) in 36% yield as a coloress solid: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.30 (br t, $J_{3,2\ (5,6)} = J_{3,4\ (5,4)} = 8.0$ Hz, 2H, aromatic H-3 and H-5), 7.08−7.00 (m, 3H, aromatic H-2, H-4 and H-6), 5.77 (br d, $J_{4,3} = 3.5$ Hz, 1H, H-4), 5.65 (dd, $J_{2,3} = 10.8$ Hz, $J_{2,1} = 3.4$ Hz, 1H, H-2), 5.53 (br d, $J_{1,2} = 2.5$ Hz, 1H, H-1), 5.26 (dd, $J_{3,2} = 10.8$ Hz, $J_{3,4} = 3.6$ Hz, 1H, H-3), 4.35 (br t, $J_{5,6a} = J_{5,6b} = 6.5$ Hz, 1H, H-5), 4.13 (dd, $J_{6a,6b} = 11.2$ Hz, $J_{6a,5} = 6.2$ Hz, 1H, H-6a), 4.07 (dd, $J_{6b,6a} = 11.2$ Hz, $J_{6b,5} = 7.1$ Hz, 1H, H-6b), 2.17 (s, 3H), 2.07 (s, 3H), 2.03 (s, 3H), 1.94 (s, 3H) (4xOAc). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.6 (2C), 170.2 (2C), 156.4, 129.8, 129.2, 123.1, 116.9 (2C), 95.9, 68.0, 67.9, 67.7, 67.3, 61.6, 20.9, 20.8, 20.8, 20.7.

The second fraction yielded the β anomer (*12*) in 24% yield as a colorless solid: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.30 (br t, $J_{3,2\ (5,6)} = J_{3,4\ (5,4)} = 8.0$ Hz, 2H, aromatic H-3 and H-5), 7.07 (br t, $J_{4,3} = J_{4,5} = 7.5$ Hz, 1H, aromatic H-4), 7.00 (br d, $J_{2,3\ (6,5)} = 8.0$ Hz, 2H, aromatic H-2 and H-6), 5.49 (dd, $J_{2,3} = 10.4$ Hz, $J_{2,1} = 8.0$ Hz, 1H, H-2), 5.46 (br d, $J_{4,3} = 3.0$ Hz, 1H, H-4), 5.10 (dd, $J_{3,2} = 10.4$ Hz, $J_{3,4} = 3.4$ Hz, 1H, H-3), 5.04 (d, $J_{1,2} = 8.0$ Hz, 1H, H-1), 4.24 (dd, $J_{6a,6b} = 11.3$ Hz, $J_{6a,5} = 7.0$ Hz, 1H, 6a), 4.14 (dd, $J_{6b,6a} = 11.3$ Hz, $J_{6b,5} = 6.3$ Hz, 1H, H-6b), 4.06 (br t, $J_{5,6a} = J_{5,6b} = 6.5$ Hz, 1H, H-5), 2.18 (s, 3H), 2.07 (s, 3H), 2.06 (s, 3H), 2.01 (s, 3H) (4xOAc). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.5, 170.4, 170.3, 169.5, 157.1, 129.7 (2C), 123.4, 117.1 (2C), 99.8, 71.1, 71.0, 68.8, 67.0, 61.5, 20.9, 20.8, 20.8, 20.7.

Synthesis of 4-Methylphenyl Tetra-O-*acetyl*-α-D-*galactopyranosides*. Glycosylation of penta-*O*-acetyl-β-D-galactopyranose with *p*-cresol was carried out similarly. The product was purified by flash column chromatography with hexanes−

EtOAc (4:1, v/v) to yield the α anomer (*9*) in 27% yield as a colorless solid: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.07 (br d, J = 8.5 Hz, 2H) and 6.93 (br d, J = 8.5 Hz, 2H) (4 x aromatic H), 5.70 (br d, $J_{4,3}$ = 3.5 Hz, 1H, H-4), 5.55 (dd, $J_{2,3}$ 10.8 Hz, $J_{2,1}$ = 3.4 Hz, 1H, H-2), 5.51 (br d, $J_{1,2}$ = 2.5 Hz, 1H, H-1), 5.25 (dd, $J_{3,2}$ = 10.8 Hz, $J_{3,4}$ = 3.5 Hz, 1H, H-3), 4.35 (br t, $J_{5,6a}$ = $J_{5,6b}$ = 6.5 Hz, 1H, H-5), 4.11(dd, $J_{6a,6b}$ = 11.2 Hz, $J_{6a,5}$ = 6.2 Hz, 1H, H-6a), 4.05 (dd, $J_{6a,6b}$ = 11.3 Hz, $J_{6b,5}$ = 7.1 Hz, 1 Hz, H-6b), 2.28 (s, 3H, Me), 2.15 (s, 3H), 2.09 (s, 3H), 2.00 (s, 3H), 1.93 (s, 3H) (4xOAc). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 170.5, 170.4, 170.3, 170.2, 154.3, 132.6, 130.2 (2C), 116.8 (2C), 95.3, 68.1 (2C), 68.0, 67.1 (2C), 61.2, 20.9, 20.8, 20.8, 20.7.

Synthesis of Cyclohexyl α-D-*Galactopyranoside.* Cyclohexyl tetra-*O*-acetyl-α-D-galactopyranoside (103 mg, 0.24 mmol) was treated with 0.01M NaOMe in anhydrous MeOH (5 mL) in an ice bath overnight. The reaction mixture was neutralized with Amberlite IR-120 (H^+) resin. The resin was removed by filtration and the filtrate concentrated in vacuo to yield the title compound (59 mg, 94%) as a colorless solid: ^{1}H NMR (D_2O, 500 MHz) δ 5.08 (d, $J_{1,2}$ = 3.9 Hz, 1H, H-1), 4.00 (br t, $J_{5,6a}$ = $J_{5,6b}$ = 6.5 Hz, 1H, H-5), 3.95 (br d, $J_{4,3}$ = 3.0 Hz, 1H, H-4), 3.83 (dd, $J_{3,2}$ = 10.3 Hz, $J_{3,4}$ = 3.3 Hz, 1H, H-3), 3.77 (dd, $J_{2,3}$ = 10.3 Hz, $J_{2,1}$ = 3.9 Hz, 1H, H-2), 3.71 (br d, J = 6.0 Hz, 2H, H-6a and H-6b), 3.58−3.68 (m, 1H, cyclohexyl H-1), 1.94−1.14 (m, 10H, cyclohexyl 5 x CH_2). ^{13}C NMR (MeOH-d_4, 100 MHz) δ 97.5, 77.6, 77.8, 70.5, 70.3, 69.3, 62.1, 34.0, 32.0, 26.1, 25.0, 24.8.

Synthesis of Cyclohexyl β-D-*Galactopyranoside.* De-*O*-acetylation of cyclohexyl tetra-*O*-acetyl-β-D-galactopyranoside in a similar manner yielded the title compound (*13*) in 80% yield as a colorless solid: ^{1}H NMR (D_2O, 400 MHz) δ 4.50 (d, $J_{1,2}$ = 8.0 Hz, 1H, H-1), 3.90 (br d, $J_{4,3}$ = 3.0 Hz, 1H, H-4), 3.65−3.69 (m, 4H, H-5, H-6a, H-6b, and cyclohexyl H-1), 3.62 (dd, $J_{3,2}$ = 9.9 Hz, $J_{3,4}$ = 3.5 Hz, 1H, H-3), 3.46 (dd, $J_{2,3}$ = 9.9 Hz, $J_{2,1}$ = 8.0 Hz, 1H, H-2), 1.96−1.14 (m, 10H, cyclohexyl 5 x CH_2). ^{13}C NMR (MeOH-d_4, 100 MHz) δ 100.7, 78.6, 74.9, 72.8, 70.7, 68.5, 60.736, 32.9, 31.3, 24.9, 23.798, 23.6.

Synthesis of Phenyl α-D-*Galactopyranoside.* A similar de-*O*-acetylation of phenyl tetra-*O*-acetyl-α-D-galactopyranoside yielded the title compound (*11*) in a quantitative yield as a colorless solid: ^{1}H NMR (D_2O, 400 MHz) δ 7.40 (br t, $J_{3,2\ (5,6)}$ = $J_{3,4\ (5,4)}$ = 8.0 Hz, 2H, aromatic H-3 and H-5), 7.18 (br d, $J_{2,3\ (6,5)}$ = 8.0 Hz, 2H, aromatic H-2 and H-6), 7.13 (br t, $J_{4,3}$ = $J_{4,5}$ = 8.0 Hz, 1H, aromatic H-4), 7.00 (7.18 (−7.14 (m, 5H, 5 x aromatic H), 5.68 (d, $J_{1,2}$ = 3.8 Hz, 1H, H-1), 4.09 (dd, $J_{3,2}$ = 10.2 Hz, $J_{3,4}$ = 3.3 Hz, 1H, H-3), 4.06 (m, 2H, H-4 and H-5), 3.99 (dd, $J_{2,3}$ = 10.2 Hz, $J_{2,1}$ = 3.8 Hz, 1H, H-2), 3.70 (br d, $J_{6a,5}$ = $J_{6b,5}$ = 6.1 Hz, 2H, H-6a and H-6b). ^{13}C NMR (MeOH-d_4, 100 MHz) d 157.2, 130.8 (2C), 124.0, 118.3 (2C), 98.4, 72.6, 70.483, 70.1, 69.1, 61.9.

Synthesis of Phenyl β-D-*Galactopyranoside.* A similar de-*O*-acetylation of phenyl tetra-*O*-acetyl-β-D-galactopyranoside yielded the title compound (*12*) in a quantitative yield as a colorless solid: ^{1}H NMR (D_2O, 400 MHz) δ 7.39 (br t, $J_{3,2\ (5,6)}$ = $J_{3,4\ (5,4)}$ = 8.0 Hz, 2H, aromatic H-3 and H-5), 7.16−7.10 (m, 3H, aromatic H-2, H-4 and H-6), 5.07 (d, $J_{1,2}$ = 7.4 Hz, 1H, H-1), 4.00 (br d, $J_{4,3}$ = 3.0 Hz, 1H, H-4), 3.86 (br t, $J_{5,6a}$ = $J_{5,6b}$ = 6.5 Hz, 1H, H-5), 3.83−3.74 (m, 4H, H-2, H-3, H-6a and H-6b). ^{13}C NMR (MeOH-d_4, 100 MHz) δ 157.8, 130.8 (2C), 124.1 (2C), 117.5, 101.8, 76.4, 73.6, 71.6, 69.5, 61.7.

Synthesis of 4-Methylphenyl α-D-*Galactopyranoside.* 4-Methylphenyl tetra-*O*-acetyl-α-D-galactopyranoside was similarly de-*O*-acetylated to yield the title compound (*9*) in 98% yield as a colorless solid: ^{1}H NMR (D_2O, 500 MHz) d 7.21 (d, J = 8.6 Hz, 2H) and 7.08 (d, J = 8.6 Hz, 2H) (4 × aromatic H), 5.60 (d, $J_{1,2}$ = 3.8 Hz, 1H, H-1), 4.09−4.04 (m, 3H, H-3, H-4, H-5), 3.97 (dd, $J_{2,3}$ = 9.8 Hz, $J_{2,1}$ = 3.9 Hz, 1H, H-2), 3.69 (br d, $J_{6a,5}$ = $J_{6b,5}$ = 6.5 Hz, 2H, H-6a and H-6b). ^{13}C NMR (MeOH-d_4, 125 MHz) δ 155.3, 133.7, 131.1 (2C), 118.3 (2C), 98.9, 72.6, 70.6, 70.2, 69.2, 61.9, 20.6.

Growth of Cells and Preparation of Right-Side-Out (RSO) Membrane Vesicles. E. coli T184 expressing single-Cys148 permease with a biotin-acceptor domain at the C terminus (*14*) were grown in Luria-Bertani broth, and RSO membrane vesicles were prepared as described previously (*5*, *15*, *16*). 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 (*5*, *6*, *17*, *18*). Single-Cys148 permease which was used for the assays contains a biotin-acceptor domain at the C terminus and is biotinylated in vivo. RSO membrane vesicles were pre-equilibrated in a final volume of 50 μL (containing 0.6 mg protein) with given sugar concentrations for 5 min at room temperature. Labeling was initiated by addition of 12 μL of [^{14}C]NEM (40 mCi/mmol) to a final concentration of 0.5 mM, and the vesicles were incubated for 5 min at room temperature (ca. 24 °C). 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 equilibration buffer, and biotinylated permease was then eluted with 50 μL of equilibration buffer containing 5 mM (+)-biotin. After addition of 25 μL of electrophoresis sample buffer (concentrated 5×), 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 (*5*, *19*).

RESULTS

Synthesis and Binding of Cyclohexyl and Phenyl α-D-*Galactopyranosides.* Previous observations indicate that hydrophobic substituents as small as a methyl group in α anomeric configuration significantly increase binding affinity of D-galactopyranosides to lac permease (*5*). To examine whether larger hydrophobic aglycons yield sugars with better affinities, cyclohexyl and phenyl derivatives of D-galactose were synthesized. Binding of sugars to lac permease was assessed by ligand-dependent protection of Cys148 against

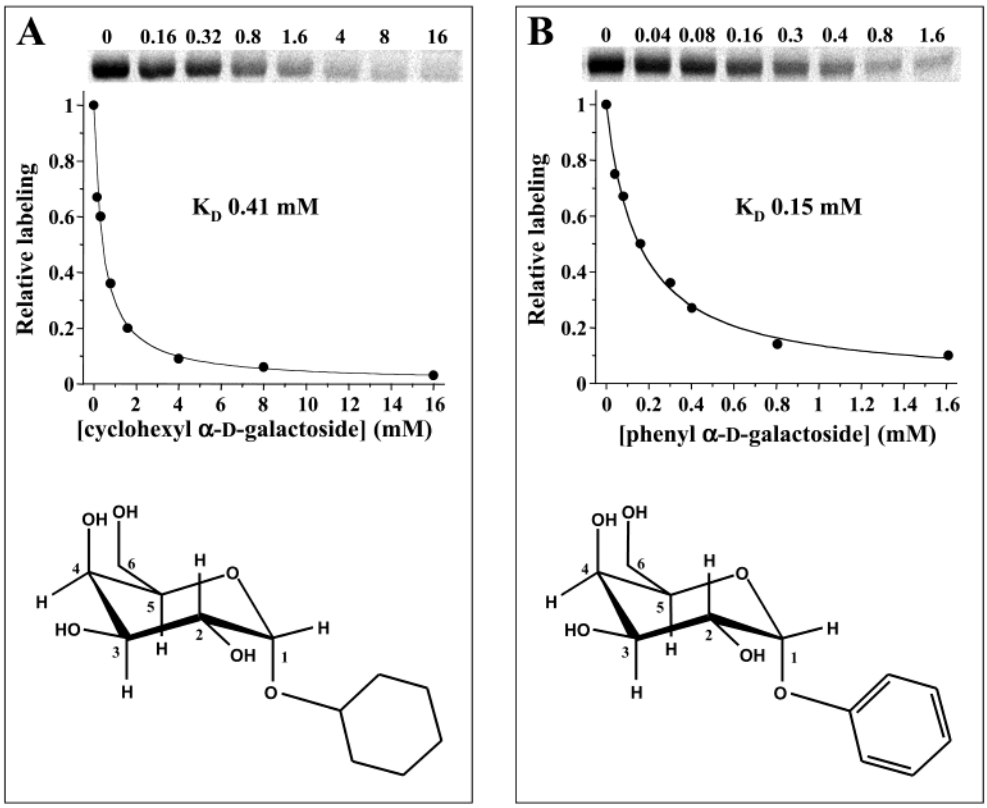

FIGURE 1: Substrate−protection against [^{14}C]NEM alkylation of Cys148 by cyclohexyl (A) and phenyl (B) α-D-galactopyranosides. RSO membrane vesicles containing single-Cys148 permease with a biotin-acceptor domain at the C terminus were incubated in 100 mM KP_i (pH 7.5) with 0.5 mM [^{14}C]NEM for 5 min in the absence or presence of the indicated concentrations of sugars. Reactions were quenched with DTT, and biotinylated permease was solubilized and purified by affinity chromatography on monomeric avidin. Samples were separated on a $NaDodSO_4$−12% 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 sugar is expressed as % labeling observed in the absence of sugar.

Table 1: Apparent Binding Affinities of D-Galactose and Hydrophobic α and β-D-Galactopyranosides to Lac Permease

compound	K_D (mM)	ref
D-galactose	30	*5*
methyl α-D-galactopyranoside	0.5	*5*
allyl α-D-galactopyranoside	0.5	*5*
cyclohexyl α-D-galactopyranoside	0.41	this work, Figure 1A
phenyl α-D-galactopyranoside	0.15	this work, Figure 1B
o-nitrophenyl α-D-galactopyranoside	0.067	this work, Figure 2A
m-nitrophenyl α-D-galactopyranoside	0.047	this work, Figure 2B
p-nitrophenyl α-D-galactopyranoside	0.014	*6*
p-methylphenyl α-D-galactopyranoside	0.019	this work, Figure 3A
α-naphthyl α-D-galactopyranoside	0.040	this work, not shown
methyl β-D-galactopyranoside	10	*5*
methyl 1-thio-β-D-galactopyranoside	3.7	this work, not shown
isopropyl β-D-galactopyranoside	1	this work, Figure 4D
cyclohexyl β-D-galactopyranoside	2.3	this work, Figure 4A
phenyl β-D-galactopyranoside	1.3	this work, Figure 4B
p-nitrophenyl β-D-galactopyranoside	1.2	this work, Figure 4C
o-nitrophenyl β-D-galactopyranoside	1.5	this work, not shown

alkylation by [^{14}C]NEM. Binding affinity of cyclohexyl α-D-galactopyranoside (K_D = 0.41 mM, Figure 1A, Table 1) was comparable to the K_D determined previously for methyl α-D-galactopyranoside, indicating that the extended hydrophobic surface of the aglycon does not result in stronger interactions with the permease. On the other hand, it has been long known that *p*-nitrophenyl α-D-galactopyranoside binds with high affinity (see Figure 3, Table 1) (*20*), suggesting that the phenyl ring plays an important role in binding. To test this notion, phenyl α-D-galactopyranoside was synthesized, and binding affinity was determined (K_D = 0.15 mM, Figure 1B, Table 1). The result indicates that a phenyl ring per se confers only a modest gain in binding affinity relative to a cyclohexyl or methyl moiety.

Binding of α-D-Galactopyranosides with Substituted Phenyl Rings. The previously determined (*6*) equilibrium dissociation constant for *p*-nitrophenyl α-D-galactopyranoside binding (0.014 mM, see Figure 3B, Table 1) is at least 10-fold lower than the K_D for phenyl α-D-galactopyranoside, suggesting that substituted phenyl rings are more effective in increasing binding affinity. Indeed, both *o*- and *m*-nitrophenyl α-D-galactopyranosides bind somewhat better than phenyl α-D-galactopyranoside, albeit with affinities (K_D = 0.067 mM and 0.047 mM, respectively) that are lower than that of *p*-nitrophenyl α-D-galactopyranoside (Figure 2, Table 1). In contrast, *p*-methylphenyl α-D-galactopyranoside (K_D = 0.019 mM) binds almost as well as *p*-nitrophenyl α-D-galactopyranoside (Figure 3, Table 1). Finally, α-naphthyl α-D-galactopyranoside which contains a bicyclic aromatic ring binds with a K_D of 0.04 mM (data not shown, Table 1).

Binding of β-D-Galactopyranosides. Binding affinities of β-D-galactopyranosides with various hydrophobic aglycons are also significantly increased relative to those of D-galactose; however, there appears to be no preference for a particular structure or functional group. The smaller methyl β-D-galactopyranoside or methyl 1-thio-β-D-galactopyranoside bind weakly (K_D = 10 mM (*5*) or 3.4 mM (data not

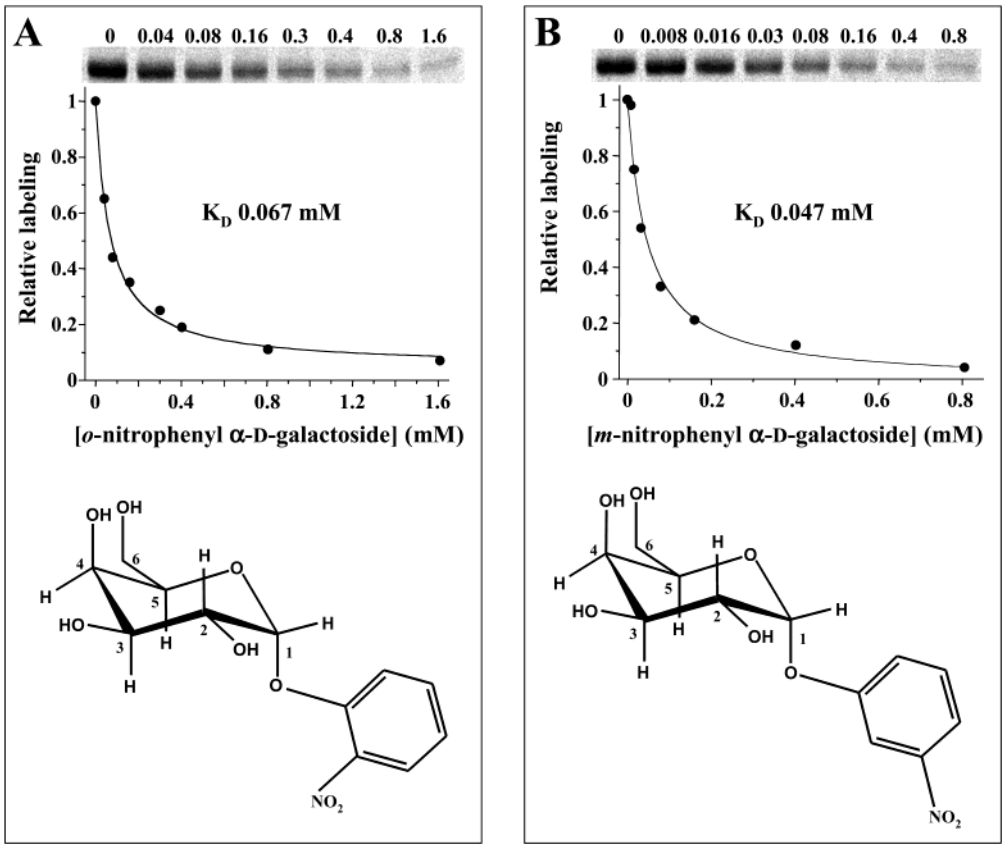

FIGURE 2: Effect of (A) *o*- and (B) *m*-nitrophenyl α-D-galactopyranosides on NEM labeling of Cys148. RSO membrane vesicles were incubated in 100 mM KP_i (pH 7.5) with 0.5 mM [^{14}C]NEM, for 5 min in the absence or presence of the indicated sugar at given concentrations. Reactions were quenched with DTT and samples were processed as described in Figure 2.

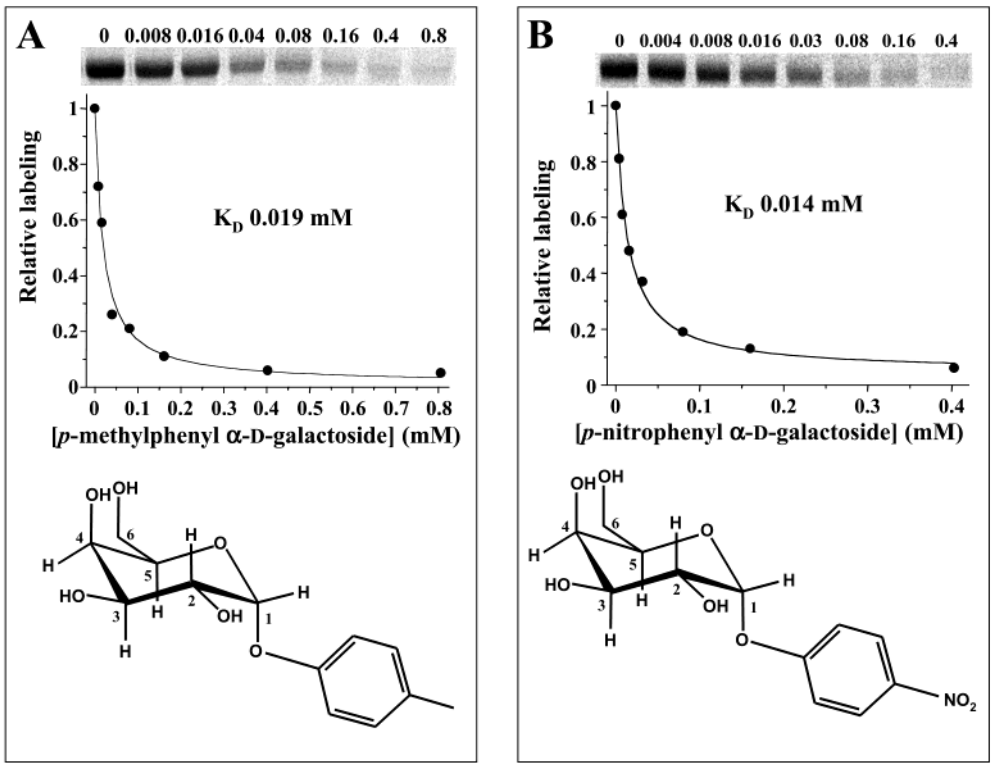

FIGURE 3: Effect of *p*-methylphenyl α-D-galactopyranoside (A) on NEM labeling of Cys148. See Figures 1 and 2 for experimental conditions. For comparison, protection data by *p*-nitrophenyl α-D-galactopyranoside (B) from ref *6* are shown.

shown, Table 1), respectively), but β-D-galactopyranosides with bulkier substituents exhibit K_D values that are fairly uniform and fall between 1 and 2.3 mM (Figure 4, Table 1).

β-D-Galactopyranosides always bind weaker than their respective α counterparts. Since α sugars show more significant variations in their binding affinities, comparing

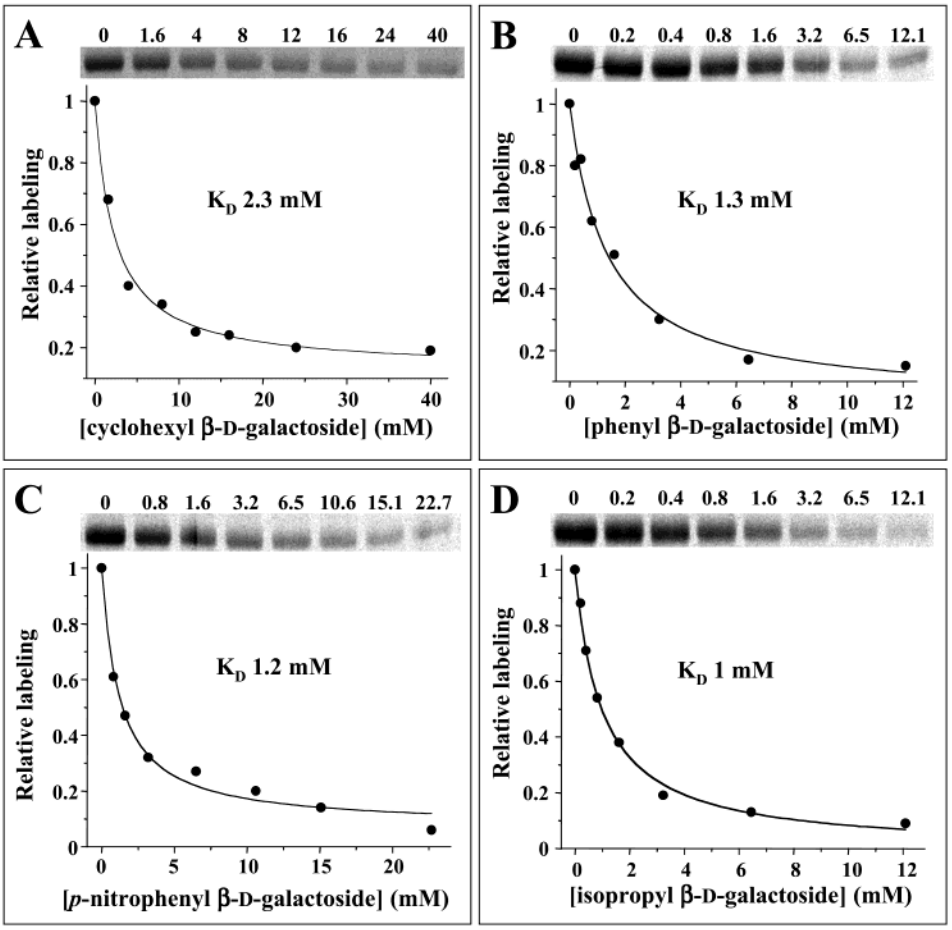

FIGURE 4: Effect of hydrophobic β-D-galactopyranosides on NEM labeling of Cys148. See Figures 1 and 2 for experimental conditions.

various α and β sugar pairs yields a range of different α/β affinity ratios, the highest observed for the *p*-nitrophenyl D-galactopyranoside pair (86-fold) and the lowest for the cyclohexyl D-galactopyranoside pair (6-fold).

DISCUSSION

The present study uses a systematic approach to examine the structural requirements of hydrophobic aglycons of D-galactopyranosides that confer high-affinity binding to lac permease. Previous observations (*5*) indicate that a single methyl group increases binding affinity by 60-fold, but only in the α anomeric configuration. In addition, *p*-nitrophenyl α-D-galactopyranoside binds with an affinity that is more than 2000-fold higher than that of D-galactose binding (*5*, *6*, *20*). On the basis of these data, we hypothesized that there is a direct correlation between the size of the hydrophobic aglycon and binding affinity to the permease, thus incrementally increasing the size of the aglycon should lead to stepwise increases in binding affinities. This hypothesis was tested first by synthesizing an α-D-galactopyranoside with a six-member aliphatic cyclic ring. Surprisingly, binding affinity of cyclohexyl α-D-galactopyranoside is practically identical to that of methyl α-D-galactopyranoside. Subsequently, an α-D-galactopyranoside with a six-member aromatic ring was synthesized (phenyl α-D-galactopyranoside) which exhibits only moderately increased affinity relative to methyl or cyclohexyl α-D-galactopyranoside. On the other hand, *o*- or *m*-nitrophenyl α-D-galactopyranosides bind with increased affinities, and the strongest binding is observed with α-D-galactopyranosides containing a *p*-substituted phenyl ring.

Taken together with previous results (*5*, *6*), the present observations indicate that the primary hydrophobic interaction between the aglycon and lac permease is directed toward the C atom closest to the galactosyl moiety, i.e., the carbon bonded to the anomeric oxygen. This conclusion derives support primarily from the observation that methyl, allyl, and cyclohexyl α-D-galactopyranosides exhibit essentially identical affinities. On the other hand, phenyl α-D-galactopyranoside binds 3-fold better, and nitro- or methyl-substituted phenyl α-D-galactopyranosides exhibit further increased binding affinities. While it appears from the results that a phenyl ring per se can provide additional binding energy, the reason substituents enhance binding is not readily apparent. Participation of the functional groups on the phenyl ring in direct interactions with the permease seems unlikely because *p*-methylphenyl or *p*-nitrophenyl α-D-galactopyranosides bind with equal efficacy. It is conceivable, however, that the plane of differently substituted phenyl rings assumes slightly different positions with respect to the galactosyl moiety, thus resulting in stronger interactions with the permease. In addition, distortion of the aromatic π ring by the functional groups might also contribute to the strength of the interaction between the phenyl ring and the permease.

Compared to α-D-galactopyranosides, binding of β-D-galactopyranosides exhibits distinct characteristics. Although binding affinity of the sugars tested here is increased 15−30-fold relative to that of D-galactose, the physicochemical

properties of the aglycon does not seem to influence binding as significantly as in the case of α-D-galactopyranosides. Thus, binding affinities between 1 and 2.3 mM were measured for the 5 β-D-galactopyranosides studied here (Table 1), which contain aglycon structures as different as isopropyl or nitrophenyl. On the basis of the model presented for the binding of α-D-galactopyranosides (see above), it seems reasonable to speculate that the generally lower binding affinities of β-D-galactopyranosides is the result of the somewhat removed position of the first carbon atom of the hydrophobic aglycon. Consequently, the hydrophobic interactions that bind α-D-galactopyranosides relatively strongly, will be weakened with respect to binding of β-D-galactopyranosides. On the other hand, it is also noteworthy that two previously studied β sugars, dansyl β-D-galactopyranoside(s) (*21*) and β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (*18*, *19*), bind with equilibrium constants that resemble those of the high-affinity α-D-galactopyranosides. While an explanation for this discrepancy is not readily apparent, these two sugars appear to be exceptions to the rule, as most β-D-galactopyranosides studied to date exhibit low binding affinities.

An interesting implication of the model is that it predicts which amino acid side-chains might participate in the hydrophobic interaction. One clear candidate is Cys148 in helix V, which interacts with the hydrophobic surface of the galactosyl moiety (*4*, *22*). Given the size of the pyranose ring, Cys148 is very likely to be in close proximity to the first C atom of the aglycon, particularly if it is in the α anomeric configuration. Another candidate is Ala122 in helix IV, which is located next to Cys148 and is also probably within 4 Å of the first C atom of the aglycon. Recent studies (*23*, *24*) show that Ala122 interacts primarily with the nongalactosyl end of disaccharide substrates of the permease. Thus, alkylation of the single Cys residue in mutant A122C with NEM selectively abolishes lactose transport, while galactose transport is relatively unaffected (*24*). Similarly, replacements of Ala122 with bulky residues (Tyr, Phe) selectively diminish disaccharide transport. It appears that Ala122 and Cys148 at the interface of helices IV and V form a hydrophobic pocket, which accommodates the hydrophobic surface of the galactosyl moiety and the first carbon of the hydrophobic aglycon. Interestingly, to date no aromatic residue has been identified in the substrate binding site of lac permease. This stands in contrast with soluble carbohydrate binding proteins, where aromatic stacking of the sugar ring by Trp or Phe side-chains is routinely observed. One possible candidate for aromatic interactions is Trp151, located one turn away from Cys148 in helix V. Replacement of Trp151 with Cys abolishes transport activity (*25*), while substitution with Phe results in normal activity, but binding was not studied (*26*). Experiments are currently underway to determine whether a direct interaction exists between Trp151 and bound D-galactopyranosides.

REFERENCES

1. Kaback, H. R., Sahin-Tóth, M., and Weinglass, A. B. (2001) *Nat. Rev. Mol. Cell Biol 2*, 610−622.
2. Sandermann, H., Jr. (1977) *Eur. J. Biochem. 80*, 507−515.
3. Olsen, S. G., and Brooker, R. J. (1989) *J. Biol. Chem. 264*, 15982−15987.
4. Wu, J., and Kaback, H. R. (1994) *Biochemistry 33*, 12166−12171.
5. Sahin-Tóth, M., Akhoon, K. M., Runner, J., and Kaback, H. R. (2000) *Biochemistry 39*, 5907−5103.
6. Sahin-Tóth, M., Lawrence, M. C., Nishio, T., and Kaback, H. R. (2001) *Biochemistry 40*, 13015−13019.
7. Still, W. C., Kahn, M., and Mitra, A. (1978) *J. Org. Chem. 43*, 2923−2925.
8. Banoub, J., and Bundle, D. R. (1979) *Can. J. Chem. 57*, 2085−2090.
9. Dey, P. M. (1967) *Chem. Ind. (London)* 1637.
10. Schroeder, L. R., Counts, K. M., and Haigh, F. C. (1974) *Carbohydr. Res. 37*, 368−372.
11. Tsuzuki, Y., Koyama, M., Aoki, K., Kato, T., and Tanabe, K. (1969) *Bull. Chem. Soc. Jpn. 42*, 1052−1059.
12. Helferich, B., and Schmitz-Hillebrecht, E. (1933) *Chem. Ber. 66*, 378−383.
13. de Bruyne, C. K., and van der Groen, G. (1972) *Carbohydr. Res. 25*, 59−65.
14. Consler, T. G., Persson, B. L., Jung, H., Zen, K. H., Jung, K., Prive, G. G., Verner, G. E., and Kaback, H. R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6934−6938.
15. Kaback, H. R. (1971) *Methods Enzymol. 22*, 99−120.
16. Short, S. A., Kaback, H. R., and Kohn, L. D. (1975) *J. Biol. Chem. 250*, 4291−4296.
17. Frillingos, S., and Kaback, H. R. (1996) *Biochemistry 35*, 3950−3956.
18. Sahin-Tóth, M., le Coutre, J., Kharabi, D., le Maire, G., Lee, J. C., and Kaback, H. R. (1999) *Biochemistry 38*, 813−819.
19. Sahin-Tóth, M., Karlin, A., and Kaback, H. R. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 10729−10732.
20. Rudnick, G., Schuldiner, S., and Kaback, H. R. (1976) *Biochemistry 15*, 5126−5131.
21. Schuldiner, S., Weil, R., and Kaback, H. R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 109−112.
22. Jung, H., Jung, K., and Kaback, H. R. (1994) *Biochemistry 33*, 12160−12165.
23. Kwaw, I., Zen, K.-C., Hu, Y., and Kaback, H. R. (2001) *Biochemistry 40*, 10491−10499.
24. Guan, L., Sahin-Tóth, M., and Kaback, H. R. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 6613−6618.
25. Weitzman, C., and Kaback, H. R. (1995) *Biochemistry 34*, 2310−2318.
26. Menezes, M. E., Roepe, P. D., and Kaback, H. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1638−1642.

BI0203076