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Sugar Recognition by CscB and LacY

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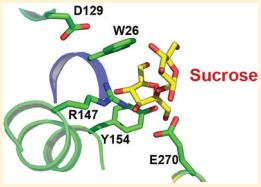
ABSTRACT: The sucrose permease (CscB) and lactose permease (LacY) of *Escherichia coli* belong to the oligosaccharide/H^+ symporter subfamily of the major facilitator superfamily, and both catalyze sugar/H^+ symport across the cytoplasmic membrane. Thus far, there is no common substrate for the two permeases; CscB transports sucrose, and LacY is highly specific for galactopyranosides. Determinants for CscB sugar specificity are unclear, but the structural organization of key residues involved in sugar binding appears to be similar in CscB and LacY. In this study, several sugars containing galactopyranosyl, glucopyranosyl, or fructofuranosyl moieties were tested for transport with cells overexpressing either CscB or LacY. CscB recognizes not only sucrose but also fructose and lactulose, but glucopyranosides are not transported and do not inhibit sucrose transport. The findings indicate that CscB exhibits practically no specificity with respect to the glucopyranosyl moiety of sucrose. Inhibition of sucrose transport by CscB tested with various fructofuranosides suggests that the C_3-OH group of the fructofuranosyl ring may be important for recognition by CscB. Lactulose is readily transported by LacY, where specificity is directed toward the galactopyranosyl ring, and the affinity of LacY for lactulose is similar to that observed for lactose. The studies demonstrate that the substrate specificity of CscB is directed toward the fructofuranosyl moiety of the substrate, while the specificity of LacY is directed toward the galactopyranosyl moiety.



Bacterial sugar transporters homologous to the lactose permease of *Escherichia coli* (LacY) belong to the oligosaccharide/H^+ symporter (OHS) subfamily of the major facilitator superfamily (MFS),[1] and LacY is the best-characterized member. Like LacY, other members of the OHS are believed to catalyze coupled translocation of a sugar and an H^+ (sugar/H^+ symport). These proteins are also likely to have a structure similar to that of LacY[2,3] with 12 mostly irregular transmembrane α-helices that traverse the membrane in zigzag fashion connected by hydrophilic loops with both N- and C-termini on the cytoplasmic face and a large water-filled cavity facing the cytoplasm.[4−6]

The second most well studied symporter in the OHS is the sucrose permease of *E. coli* (CscB), encoded by the *cscB* gene, which transports sucrose, but not lactose or other galactopyranosides.[7,8] Another OHS symporter, the melibiose permease of *Enterobacter cloacae* (MelY), which has a high degree of sequence similarity with LacY, does not transport methyl 1-thio-β-D-galactopyranoside, a good substrate for LacY,[9] while both proteins recognize melibiose and lactose as substrates. In any case, CscB exhibits 28% sequence identity with LacY and an overall level of homology of 51%.[2,10] Most of the irreplaceable residues in LacY with respect to activity are conserved in CscB, and site-directed mutagenesis confirms their importance.[3,11,12] Moreover, homology threading of CscB with the LacY crystal structure as a template, as well as functional studies of site-directed mutants in CscB, predicts similar organizations for the sugar- and H^+-binding sites.[2,3] Glu126 (helix IV), Arg144 (helix V), and Trp151 (helix V) in LacY, which are directly involved in sugar binding, are homologous with Asp129, Arg147, and Tyr154 in CscB. Although Glu270 is one helix turn closer to the cytoplasmic side of CscB than the homologous residue Glu269 (helix VIII) in LacY, the functional role appears to be the same.[3] In addition, the spatial organization of the residues involved in H^+ translocation in LacY is almost identical in CscB, as judged from homology modeling.[2,3]

There are many bacterial genes encoding proteins that are significantly similar to LacY and CscB, although their expression and transport specificities have yet to be studied.[2] LacY specifically recognizes D-galactose and the D-galactopyranosyl moiety of its disaccharide substrates but has no affinity for D-glucopyranosides or D-glucose.[13−16] Therefore, it was assumed that the specificity of CscB would be directed at the D-glucopyranosyl moiety of sucrose. To test this notion, we identified several sugars that might hypothetically be transported by CscB and found that CscB catalyzes transport of not only sucrose but also lactulose and fructose (Figure 1). Moreover, D-glucopyranoside has no detectable affinity for CscB. Thus, the specificity of CscB is directed toward the fructofuranosyl ring of sucrose and not the glucopyranosyl moiety. In addition, the C_3-OH group on the fructofuranosyl ring appears to be important for recognition. Finally, lactulose is an excellent substrate for LacY, as predicted from its specificity for the galactopyranosyl moiety of its multiple substrates.

EXPERIMENTAL PROCEDURES

Materials. Fructose, glucose, sucrose, lactulose, turanose, and palatinose were of the highest available grade and purchased from Sigma-Aldrich. Octyl α-D-galactopyranoside was from Carbosynth Ltd. [U-^{14}C]Sucrose was purchased from

Received: October 17, 2011
Revised: November 18, 2011
Published: November 22, 2011

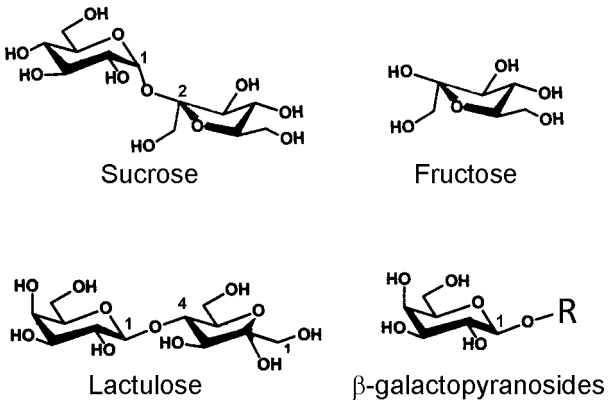


Figure 1. Chemical structures of sucrose, fructose, lactulose, and β-galactopyranosides, where R represents different anomeric moieties (e.g., in galactose, R is H; in lactose, R is glucose).

Perkin-Elmer (Boston, MA), D-[U-^{14}C]fructose from Moravek Biochemicals (Brea, CA), and [*galactose*-6-^{3}H]lactulose from American Radiolabeled Chemicals, Inc. (St. Louis, MO). DNA plasmid purification kits and the penta-His antibody–horseradish peroxidase (HRP) conjugate were obtained from Qiagen (Valencia, CA). The Supersignal West Pico Chemiluminescent substrate kit was from Pierce Inc. (Rockford, IL).

Expression Analysis. Plasmids pSP72/CscB and pT7-5/LacY were engineered to encode the appropriate permease with a C-terminal six-His tag to allow identification of protein expression by Western blot analysis. Both permeases CscB and LacY were expressed to similar levels in the membrane of *E. coli* as detected by using the penta-His HRP-conjugated antibody and Supersignal West Pico Chemiluminescent substrate.

Transport Assays. *E. coli* T184 [*lacI*$^{+}$*O*$^{+}$*Z*$^{-}$*Y*$^{-}$*(A), rspL, met*$^{-}$*, thr*$^{-}$*, recA, hsdM, hsdR/F′ lacI*^{q}*OZ*^{D118}*(Y*$^{+}$*A*$^{+}$*)*] was transformed with the appropriate expression vector and grown aerobically overnight at 37 °C in Luria-Bertani culture medium containing 100 μg/mL ampicillin. A 10-fold dilution of the culture was grown for 2 h before induction with 1 mM IPTG. Following induction, growth was continued for a further 2 h, after which the cells were harvested by centrifugation, washed with 100 mM potassium phosphate (KP_i; pH 7.0) and 10 mM $MgSO_4$, and adjusted to an absorbance at 420 nm (A_{420}) of 20 (approximately 1.4 mg/mL protein) for transport measurements. Transport of a given radiolabeled sugar was assayed at room temperature in the absence or presence of given unlabeled sugars by rapid filtration as described previously.[12,17] Transport was initiated by addition of 2 μL of radiolabeled sugar to 50 μL aliquots of cells containing 70 μg of total protein and stopped by dilution followed by rapid filtration.

Rates of transport at various substrate concentrations were measured by mixing 50 μL aliquots of cells with 50 μL of radiolabeled sugars and the reactions stopped after incubation of the mixture for 1 min at room temperature. The total level of radioactivity was maintained constant in samples with different sugar concentrations. The rates of transport were estimated after correction for sugar uptake by cells carrying a vector without an inserted transporter gene. Data were analyzed by using the Michaelis–Menten equation[18] and Sigmaplot 10 (Systat Software Inc., Richmond, CA).

MIANS Labeling. The apparent affinity of purified LacY for galactosidic sugars was measured by substrate protection of Cys148 against alkylation by 2-(4′-maleimidylanilino)-naphthalene-6-sulfonic acid (MIANS) as the effect of sugar concentration on the initial rate of MIANS labeling as described previously.[19,20] The change in fluorescence was monitored at room temperature using an SLM-Aminco (Urbana, IL) 8100 spectrofluorimeter modified by OLIS, Inc. (Bogart, GA) with excitation and emission wavelengths of 330 and 415 nm, respectively. Data fitting was conducted using SigmaPlot 10 (Systat Software Inc.).

RESULTS

Transport of Sucrose or Fructose by CscB. *E. coli* cells overexpressing CscB catalyze transport of either sucrose or fructose, while cells transformed with a vector devoid of *cscB* exhibit essentially no transport of either sugar (Figure 2A,C).

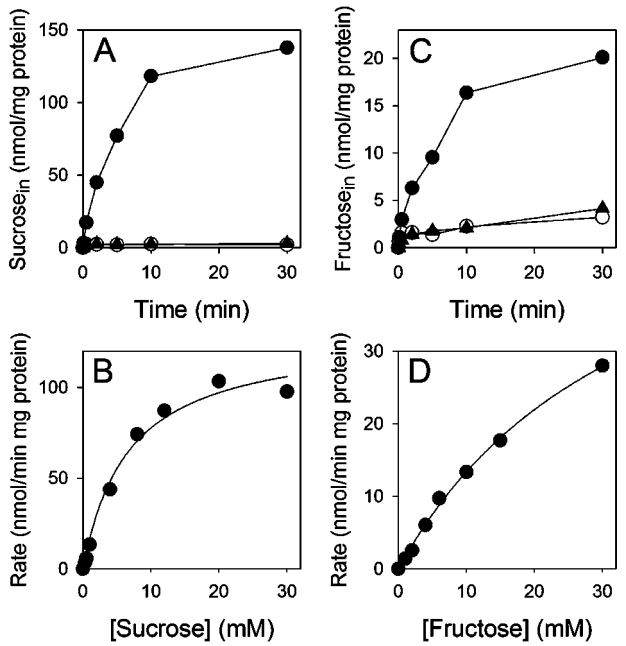


Figure 2. Transport activity of CscB. (A) Time courses of accumulation of [^{14}C]sucrose (0.5 mCi/mmol) by *E. coli* T184 cells expressing CscB (●), LacY (▲), or no permease (○) were measured at 4 mM sucrose as described in Experimental Procedures. (B) Concentration dependence of the initial rates of accumulation of sucrose by *E. coli* T184 cells expressing CscB measured as described in Experimental Procedures. The hyperbolic fit is shown as a solid line with the following estimated kinetic parameters: K_m = 6.7 ± 1.3 mM, and V_{max} = 130 nmol min^{-1} (mg of protein)$^{-1}$. (C) Time courses of accumulation of 6 mM [^{14}C]-D-fructose (0.3 mCi/mmol) were measured and presented as described for panel A. (D) Kinetic analysis of the transport of fructose by CscB conducted as described for panel B with the following estimated kinetic parameters for fructose: K_m = 36 ± 4 mM, and V_{max} = 60 nmol min^{-1} (mg of protein)$^{-1}$.

The transport of sucrose by cells expressing CscB increases at a rapid rate for ~5 min and reaches a steady-state level of ~140 nmol/mg of protein within ~20 min (Figure 2A). In contrast, the same cells catalyze the transport of fructose at a relatively low rate to a steady-state level of ~20 nmol/mg (Figure 2C). Rates of transport as a function of sucrose or fructose concentration follow a hyperbolic relationship with a K_m for sucrose of 6.7 mM and a V_{max} of 130 nmol min^{-1} (mg of protein)$^{-1}$ (Figure 2B); fructose transport exhibits a K_m of 36 mM and a V_{max} of 60 nmol min^{-1} (mg of protein)$^{-1}$ (Figure

2D). Transport of neither sucrose nor fructose by cells overexpressing LacY is observed [Figure 2A,C (▲)].

The transport of sucrose by CscB is not inhibited to any degree whatsoever by glucose because kinetic parameters of transport remain unchanged even in the presence of 30 mM glucose (Figure 3). Moreover, sugars devoid of a fructose

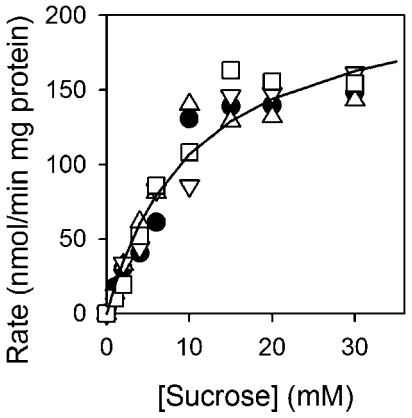


Figure 3. Effect of glucose on the transport of sucrose by CscB. Initial rates of accumulation of [^{14}C]sucrose by *E. coli* T184 cells expressing CscB were measured and fitted as described in the legend of Figure 2B in the absence of glucose (●) or in the presence of 10 (△), 20 (▽), or 30 mM glucose (□).

moiety, which include galactose, lactose, melibiose, mannose, rhamnose, and ribose, have no effect on sucrose transport (data not shown). The results indicate that the specificity of CscB is directed toward the fructofuranosyl ring of the substrate. To further evaluate the importance of the fructose moiety, we tested different fructofuranosides for their ability to inhibit the transport of sucrose by CscB (Figure 4). The sugars tested include turanose (3-*O*-*α*-D-glucopyranosyl-D-fructose), lactulose (4-*O*-*β*-D-galactopyranosyl-D-fructose), and palatinose (6-*O*-*α*-D-glucopyranosyl-D-fructose). While 10 mM palatinose or lactulose inhibits sucrose transport, albeit relatively weakly, turanose has no effect on the transport, thereby providing suggestive evidence that the C_3-OH group on the fructofuranosyl ring may be important for recognition by CscB. Detailed analysis reveals that fructose is a competitive inhibitor of sucrose transport with a K_i of 30−50 mM (Figure 4B), which is in the same range as the K_m for fructose transport (Figure 2D).

Transport of Lactulose by CscB or LacY. *E. coli* cells overexpressing CscB catalyze the transport of lactulose (Figure 5). Accumulation of this disaccharide occurs at a relatively slow rate and does not reach a steady state even by 60 min at 20 mM lactulose. In contrast, *E. coli* cells overexpressing LacY catalyze the transport of lactulose at a rapid rate to a steady-state level of ∼85 nmol/mg of protein in approximately 3 min (Figure 6A). Kinetic parameters for the transport of lactulose by LacY estimated from the concentration dependence of the initial rate (Figure 6B) are as follows: K_m = 0.24 mM, and V_{max} = 49 nmol min^{-1} (mg of protein)$^{-1}$. These values are similar to those obtained for transport of lactose by LacY.[21]

As shown previously by substrate protection against alkylation of Cys148 in LacY, galactose or lactose exhibits an apparent affinity of 50 or 9 mM, respectively,[15] while *β*-D-galactopyranosyl-1-thio-*β*-D-galactopyranoside (TDG) or 4-nitrophenyl-*α*-D-galactopyranoside (NPG) exhibits an apparent affinity of 0.85 or 0.03 mM, respectively.[20] By using the same

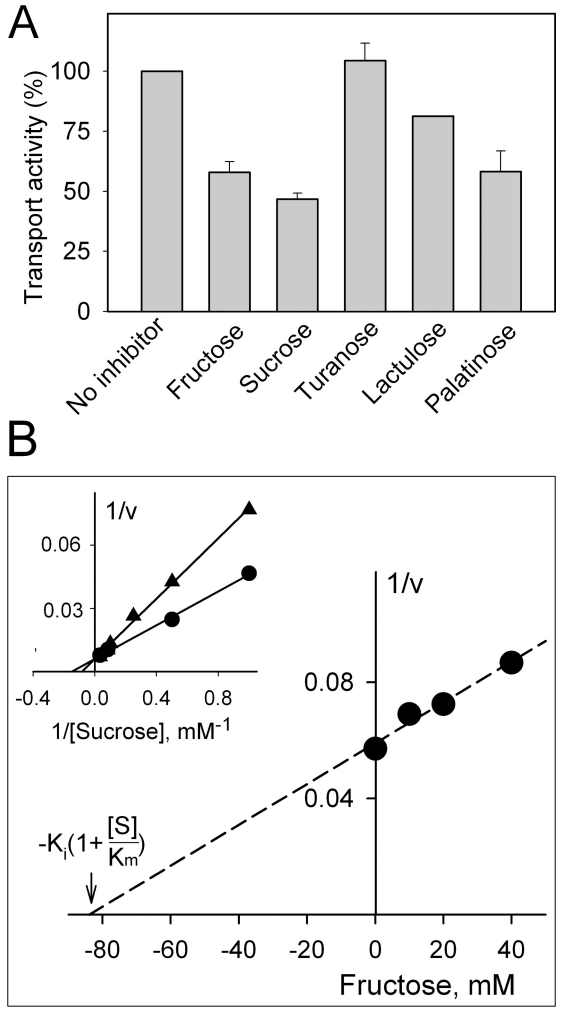


Figure 4. Effect of fructofuranosides on the transport of sucrose by CscB. (A) Testing of different sugars for their ability to inhibit the transport of sucrose. Accumulation of [^{14}C]sucrose (4 mM) by *E. coli* T184 cells expressing CscB was measured as described in the legend of Figure 2A for 10 min without additions (100% activity) or in the presence of 10 mM unlabeled fructose, sucrose, turanose, lactulose, or palatinose. (B) Concentration dependence of fructose inhibition. Initial rates of [^{14}C]sucrose transport (30 s) were measured as described for panel A at various concentrations of unlabeled fructose (10, 20, or 40 mM). The inset demonstrates competitive inhibition of the transport of [^{14}C]sucrose by 20 mM fructose (▲) with an estimated K_i of ∼30 mM and an unchanged V_{max} of 160 nmol min^{-1} (mg of protein)$^{-1}$. A Dixon plot exhibits a linear fit to the data (−−−) with an estimated K_i of ∼50 mM.

method, the apparent affinity of LacY for lactulose (K_d^{app}) is estimated to be ∼8 mM (Table 1), a value approximately the same as that observed for lactose.

■ DISCUSSION

Although there is a clear similarity in the structural organization of key residues involved in sugar binding in LacY and CscB,[2,3] as shown here, there is an unexpected and surprising difference in substrate recognition between the two symporters. Thus, CscB catalyzes the transport of sucrose, fructose, or even lactulose but exhibits no recognition of glucopyranosides, glucose in particular, as evidenced by the inability of glucose to inhibit sucrose transport. Taken together, the results lead to the conclusion that the specificity of CscB is directed toward the

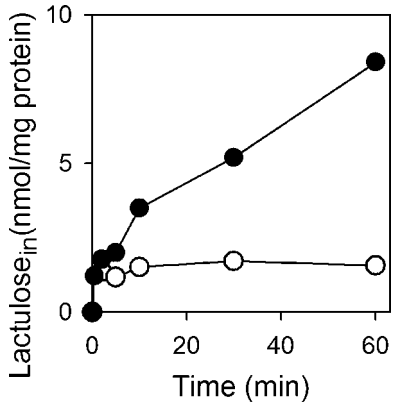


Figure 5. Lactulose transport activity of CscB. Time courses of accumulation of lactulose by *E. coli* T184 cells expressing CscB (●) or no permease (○) were measured at 20 mM [^{3}H]lactulose (0.1 mCi/mmol) as described in Experimental Procedures.

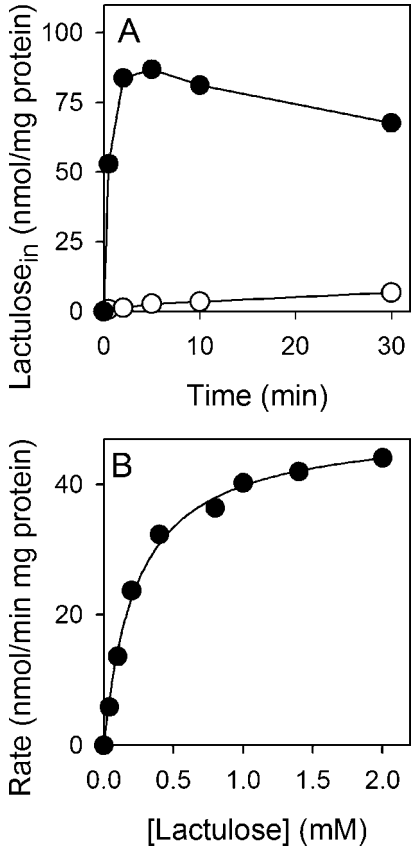


Figure 6. Lactulose transport activity of LacY. (A) Time courses of accumulation of lactulose by *E. coli* T184 cells expressing LacY (●) or no permease (○) were measured at 0.4 mM [^{3}H]lactulose (5 mCi/mmol) as described in Experimental Procedures. (B) Concentration dependence of initial rates of lactulose accumulation measured as described in Experimental Procedures. A hyperbolic fit is shown as a solid line with the following estimated kinetic parameters: K_m = 0.24 ± 0.02 mM, and V_{max} = 49 nmol min^{-1} (mg of protein)$^{-1}$.

fructofuranosyl moiety of sucrose. In contrast, extensive studies demonstrate that the specificity of LacY is directed toward the galactopyranosyl moiety of the substrate, and the C_4-OH group plays the predominant role by far in recognition and binding.[16,22] The monosaccharide galactose is the most specific substrate for LacY, although it binds with very low affinity,[13,14,22] while galactopyranosides in the α configuration with anomeric substitutions, particularly those that are hydrophobic, exhibit increased affinity with little or no effect on specificity.[16,22]

Table 1. Sugar Binding Affinities of LacY[a]

sugar	K_d^{app} (mM)
galactose	50[b]
lactose	9[b]
lactulose	8[c]
TDG	0.85[d]
α-octylgalactoside	0.05[c]
NPG	0.03[d]

[a]Apparent K_d values for galactosidic sugars measured by substrate protection of Cys148 against alkylation by MIANS as the effect of sugar concentration on the initial rate of MIANS labeling. [b]From ref 15. [c]From this study. [d]From ref 20.

Asp129 and Arg147 in CscB, which are positioned like Glu126 and Arg144 in LacY, respectively, probably also play a direct role in sugar recognition and binding, and Tyr154 in CscB, which is homologous to Trp151 in LacY, likely stacks hydrophobically with the fructofuranosyl ring of sucrose.[2,3] Glu270 in CscB, although positioned one helix turn closer to the cytoplasmic side of CscB than Glu269 in LacY, is essential and is probably also important for substrate recognition and binding. Although replacement of Ser151 (homologous to Cys148 in LacY) with Cys causes CscB to become highly sensitive to *N*-ethylmaleimide in a manner similar to that of LacY, the substrate affords no protection whatsoever against inactivation of transport or alkylation with CscB.[12] Thus, the overall architecture of the substrate-binding sites appears to be conserved in CscB and LacY; however, there are important differences in detailed interactions, and the fructofuranosyl moiety of sucrose likely occupies a position homologous to that of the galactopyranosyl moiety of lactose in LacY (Figure 7).

Inhibition studies with several fructofuranosides (Figure 4) provide some evidence that the C_3-OH group of the fructose moiety in sucrose is important for binding. In lactulose and palatinose, the galactose moiety is attached to the C_4 and C_6 atoms of the fructose moiety, respectively. These fructofuranosides partially inhibit the transport of sucrose by CscB, suggesting that the C_4-OH and C_6-OH groups are not important for binding. On the other hand, in turanose, the galactose moiety is attached to the C_3 atom of the fructose moiety, and turanose does not inhibit sucrose transport, thereby suggesting that the C_3-OH group is likely an important player in the interaction of the fructose moiety with CscB.

Transport of lactulose is catalyzed by CscB, but at a low rate. In complex with sugar binding proteins, lactulose is in an extended, planar conformation with respect to the galactose and fructose moieties, which are in the β configuration.[23] In contrast, sucrose is in a bent conformation with the glucose and fructose rings positioned at approximately a right angle (PDB entries 1AF6, 1PT2, and 1IW0). Therefore, for the C_3-OH group of the fructose moiety in a disaccharide to be maximally accessible in the sugar-binding site of CscB, the anomeric ring may need to be attached to the fructose moiety in such a manner that the C_3-OH group is readily accessible. With lactulose, the galactose ring may sterically interfere with the accessibility of the C_3-OH group of the fructose moiety, making it a relatively poor substrate for CscB. Therefore, it is clear that high-resolution crystal structures with bound substrates are

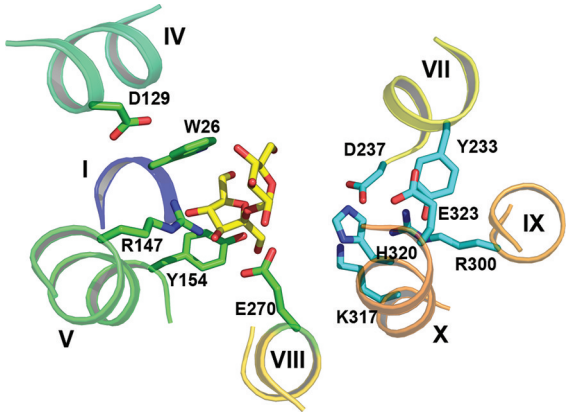


Figure 7. Sucrose molecule modeled in the putative sugar-binding site of CscB. The sugar-binding site of CscB is viewed from the cytoplasmic side with a sucrose molecule docked according to the findings presented in this paper. The fructofuranosyl moiety is in close contact with amino acid residues essential for sugar binding,[3] which are in the N-terminal six-helix bundle and shown as green sticks. Residues important for H^+ translocation are located in C-terminal six-helix bundle and shown as cyan sticks. Transmembrane helices are numbered with Roman numerals. The CscB model was built by homology modeling using the X-ray structure of LacY (PDB entry 1PV7) as a template.[2] The sucrose molecule (coordinates from PDB entry 1IW0) is presented as yellow sticks. The figure was generated with Pymol 1.3.

needed to identify critical contacts of side chains with ligands that determine binding specificity.

In contrast to CscB, lactulose is an excellent substrate for LacY. In lactulose, the galactose moiety is bonded to fructose by a β-1,4-glycosidic bond, making the OH groups at each position on the galactose moiety as accessible as in lactose. Moreover, both protein-bound lactose (PDB entries 1DLL and 1ULC) and lactulose are in extended conformations. Although the C_4-OH group is the major determinant for recognition and binding by LacY, each OH group makes a contribution to binding affinity.[16] Thus, it is not surprising that lactulose is a good substrate for LacY.

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Funding

This work was supported by National Institutes of Health Grants DK51131, DK069463, and GM074929 to H.R.K.

ACKNOWLEDGMENTS

We thank Miklós Sahin-Tóth for reading the manuscript.

ABBREVIATIONS

CscB, sucrose/H^+ symporter; LacY, lactose/H^+ symporter; OHS, oligosaccharide/H^+ symporter; MFS, major facilitator superfamily; IPTG, isopropyl 1-thio-β-D-galactopyranoside; TDG, β-D-galactopyranosyl-1-thio-β-D-galactopyranoside; NPG, 4-nitrophenyl-α-D-galactopyranoside; PDB, Protein Data Bank.

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