Substrate Recognition at the Cytoplasmic and Extracellular Binding Site of the Lactose Transport Protein of *Streptococcus thermophilus*

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The lactose transport protein (LacS) of *Streptococcus thermophilus* catalyzes the uptake of lactose in an exchange reaction with intracellularly formed galactose. The interactions between the substrate and the cytoplasmic and extracellular binding site of LacS have been characterized by assaying binding and transport of a range of sugars in proteoliposomes, in which the purified protein was reconstituted with a unidirectional orientation. Specificity for galactoside binding is given by the spatial configuration of the C-2, C-3, C-4, and C-6 hydroxyl groups of the galactose moiety. Except for a C-4 methoxy substitution, replacement of the hydroxyl groups of the galactose moiety. Large hydrophobic or hydrophilic substitutions on the galactose C-1 α or β position did not impair transport. In fact, the hydrophobic groups increased the binding affinity but decreased transport rates compared with galactose. Binding and transport characteristics of deoxygalactosides from either side of the membrane showed that the cytoplasmic and extracellular binding site interact differently with galactose. Compared with galactose, the IC₅₀ values for 2-deoxy- and 6-deoxygalactose at the cytoplasmic binding site were increased 150- and 20-fold, respectively, whereas they were the same at the extracellular binding site. From these and other experiments, we conclude that the binding sites and translocation pathway of LacS are spacious along the C-1 to C-4 axis of the galactose moiety and are restricted along the C-2 to C-6 axis. The differences in affinity at the cytoplasmic and extracellular binding site ensure that the transport via LacS is highly asymmetrical for the two opposing directions of translocation.

The lactose transport protein, LacS, of *Streptococcus thermophilus* belongs to a family of secondary transport proteins, termed GPH, that transport galactosides, pentosides, or hexuronic acids (1). Most members of the GPH family have a structural fold that is composed of 12 transmembrane segments. LacS and some other members differ from these proteins by having an additional carboxyl-terminal cytoplasmic domain of about 180 amino acids (2). This cytoplasmic domain is homologous to IIA proteins/domains of various phosphoenolpyruvate:sugar phosphotransferase systems, and its phosphorylation state influences the transport activity (3).

In *S. thermophilus* lactose is taken up via the lactose transport system, and intracellularly the disaccharide is hydrolyzed into glucose and galactose by the action of β-galactosidase. The glucose moiety is metabolized, and the galactose moiety is excreted into the medium by action of the LacS carrier. The resulting reaction catalyzed by LacS is a lactose/galactose exchange, which is driven by the concentration gradients of both sugars across the membrane (5). The LacS protein also catalyzes a galactoside/H⁺ symport, but this transport reaction is one to two orders of magnitude slower than the exchange reaction and therefore less relevant in *vivo*.

Any transport protein catalyzing an exchange or a proton symport reaction must oscillate between a minimum of two conformations, that is one in which the binding site faces toward the outside and one where the binding site faces toward the inside of the cell. *A priori*, one would expect that sugar binds with a higher affinity to the extracellular than to the cytoplasmic binding site; the latter is the site from where the sugar taken up has to be released. However, this is not necessarily true for a system like LacS; where following release of lactose, galactose is bound to the cytoplasmic binding site and subsequently released from the extracellular binding site.

Surprisingly, little is known about the interactions of a sugar with the cytoplasmic and extracellular binding site of any (sugar) transport system. Ideally, one would like to have high resolution structural information of the transport protein to define the substrate binding site as there is for sugar-binding proteins and sugar binding toxins (6–10). In these proteins sugar binding is accomplished by (i) extensive hydrogen bonding to which ordered water molecules participate and (ii) hydrophobic interactions with aromatic residues, which in some cases tightly stack the sugar ring between two or more aromatic residues. As no such information is available for sugar transport systems, an alternative approach was sought to dissect the structural requirements for substrate binding by LacS. Substrate specificity studies have proven to give valuable insight into the nature of the interactions between the sugar and the protein in case of the human sugar transporters Glut1–4 (11, 12) and their *Escherichia coli* homologues the galactose (GalP) and l-arabinose (AraE) proton symporters (13, 14), the lactose permease LacY from *E. coli* (15, 16), and the human intestinal brush border glucose/Na⁺ cotransporter SGLT1 (17), the *Trypanosoma brucei* bloodstream form transporter THTL1 (18), and the human active renal hexose transporter (19). Most of

* This work was supported by Grants B10-4-CT-960129 and 960439 from the European Community. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1744 solely to indicate this fact.

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1 M. G. W. Gunnewijk and B. Poolman, manuscript in preparation.
these studies, however, do not discriminate between sugar binding to the cytoplasmic and extracellular binding site.

In this study we report on the interactions between the substrate and the cytoplasmic and extracellular binding site of LacS by assaying for binding and transport of a range of sugars. Importantly, we are able to specifically observe both binding sites by reconstituting purified LacS to form proteoliposomes with a unidirectional orientation of the protein (20, 21).

In the proteoliposomes the extracellular binding site faces the interior of the proteoliposomes.

**EXPERIMENTAL PROCEDURES**

**Materials—**β-D-glucose-1-14C Lactose (2.11 teslabecquerel/mol) was obtained from the Radiochemical Center, Amersham Pharmacia Biotech. Ni-nitrotriacetic acid resin was from Qiagen, Inc.; Bio-Beads SM-2 were from Bio-Rad; and Triton X-100 was from Amersham Pharmacia Biotech. Total E. coli lipids and egg yolk t-α-phosphatidylcholine were obtained from Avanti Polar Lipids and Sigma, respectively. 4-O-β-D-Galactopyranosyl-β-D-glucose (melibiose), O-α-D-galactopyranosyl-(1,6)-α-D-galactopyranosyl-(1,6)-α-D-galactopyranosyl-(1,2)-β-D-fructofuranoside (stachyose), O-α-D-galactopyranosyl-(1,6)-β-D-glucopyranosyl-(1,2)-β-D-fructofuranoside (raffinose), α-t-talose, α-naphthyl α-D-galactopyranoside (α-NPG), α-naphthyl ω-b-D-galactopyranoside (ω-NPG), 2-deoxy-o-galactose, β-D-fucose, β-D-glucose, β-D-galactose, methyl-3-O-β-D-galactopyranosyl-β-D-galactopyranoside, methyl β-D-thiogalactoside (TMG), methyl-4-O-β-D-galactopyranosyl-β-D-galactopyranoside, 4-O-(2-O-methyl-β-D-galactopyranosyl)-o-D-glucopyranoside, 6-O-β-D-galactopyranoside, O-nitrophenyl β-D-galactopyranoside (ONPG), O-nitrophosphoryl α-D-galactopyranoside (α-ONPG), phenyl-β-D-galactoside (β-PG), S-β-D-galactopyranosyl-(1,1)-β-D-galactopyranoside (ONPG), and thio-β-D-thiogalactoside (ONPG) were obtained from Sigma. Isopropyl-1-thio-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl β-D-thiogalactoside (X-gal) were from Roche Molecular Biochemicals. All other materials were reagent grade and were obtained from commercial sources.

**Bacterial Strains and Growth Conditions—**S. thermophilus strain ST111lacS expressing his-tagged LacS from pGKHis was grown semi-aerobic at 42 °C in (Belliker broth (22) supplemented with 0.5% beef extract, 0.5% lactose, and 4 μg/ml erythromycin (3, 20).

**Isolation of Membranes and Purification and Reconstitution of LacS—**Right-side-out membrane vesicles of S. thermophilus were isolated as described (20, 23). Solubilization, purification using nickel affinity chromatography, and reconstitution of LacS into Triton X-100-doped liposomes yielding proteoliposomes in which the LacS to lipid ratio is 1:100 on a weight to weight basis were described previously (20).

**Counterflow—**All transport assays were carried out at 30 °C. Proteoliposomes in KPM buffer (50 mM potassium phosphate, pH 7.0, 2 mM MgSO4) plus 10 mM lactose were frozen in liquid nitrogen and thawed slowly at room temperature. After extrusion through a 400-nm filter, the proteoliposomes were centrifuged (20 min at 280,000 × g, 25 °C) and resuspended to about 1 mg/ml LacS and correspondingly 100 mg/ml lipid. To determine the apparent affinity constant for lactose at the cytoplasmic binding site (facing the outside of the proteoliposomes), an aliquot of 1 μl of proteoliposome suspension was diluted into 200 μl of KPM containing different concentrations of [14C]lactose. The uptake of [14C]lactose was stopped at different time intervals by dilution of the proteoliposome suspension into buffer without galactose addition. All data were corrected for background binding of [14C]lactose to the membranes and filters by subtracting the amount of label at time point zero from the counterflow data and by subtracting the amount of label at infinite time point from the exchange and efflux data. The amount of [14C]lactose retained inside the proteoliposomes after complete equilibration of [14C]lactose-preloaded proteoliposomes with external buffer is not significant compared with the background binding and was thus not corrected for.

The uptake of [14C]lactose in the counterflow reaction was linear in time for at least the first 16 s, and the data were analyzed by linear regression. The exchange data were fitted to a function describing an exponential decay,

\[
Y_t = A \exp^{-Bt} \quad (\text{Eq. 1})
\]

where \(Y_t\) (in nmol) is the amount of lactose inside the proteoliposomes at time point \(t\), and \(A\) (in nmol) is the amount of lactose inside the proteoliposomes at time point zero. \(B\) is the decay constant of the reaction. \(A\) was calculated by multiplying the initial sugar concentration (in nmol) with the specific internal volume of the proteoliposomes.

Initial rates were calculated from the amount of lactose inside the proteoliposomes at time point zero (\(A\)) with the decay constant (\(B\)).

The specific internal volume of the [14C]lactose preloaded proteoliposomes used in the exchange and efflux assays was estimated from the amount of radioactive label present inside the proteoliposomes at time point zero and the total amount of radioactive label present in the proteoliposome suspension (estimated 1.5 μdmg/lipid). The amount of radioactive label at time point zero was determined by extrapolation.

**RESULTS**

**Exchange and Efflux Down the Concentration Gradient—**Proteoliposomes were prepared for the uptake experiments as described for the counterflow assay. The apparent affinity constant for lactose at the extracellular binding site (facing the interior of the proteoliposomes) was determined using proteoliposomes that were preloaded with different concentrations of lactose and subsequently equilibrated for 2 h with trace amounts of [14C]lactose. At time point zero, 1 μl of proteoliposome suspension was diluted into 1 ml of KPM without (efflux) or with (exchange) 1 mM lactose (approximately four times the \(K_m^{ex} \) at the cytoplasmic binding site), and the exit of lactose was stopped at different time points as described above. \(I_{C50}\) values at the extracellular binding site were measured using proteoliposomes that were preloaded with 1 mM of [14C]lactose (approximately 0.2 times the \(K_m^{ex} \) at the extracellular binding site) plus at least six different concentrations of inhibitor. The reaction was started by dilution of the proteoliposomes into 1 ml of KPM plus 0.5 mM lactose (approximately two times the \(K_m^{ex} \) at the cytoplasmic binding site). The exit of [14C]lactose was stopped at different time points as described above. To determine whether or not galactose analogs accelerate the exit of [14C]lactose and are thus transported, proteoliposomes were preloaded with 2.5 mM [14C]lactose and diluted 200-fold into KPM with 5 mM of the galactose analog. Transport rates were compared with those of efflux transport, where proteoliposomes were diluted into buffer without galactose analog.

**Data Analysis—**All data were corrected for background binding of [14C]lactose to the membranes and filters by subtracting the amount of label at time point zero from the counterflow data and by subtracting the amount of label at infinite time point from the exchange and efflux data. The amount of [14C]lactose retained inside the proteoliposomes after complete equilibration of [14C]lactose-preloaded proteoliposomes with external buffer is not significant compared with the background binding and was thus not corrected for.

[The text continues with various chemical reactions and experimental details, including the use of various sugars and inhibitors to study the transport properties of LacS.]
assay exit of \([^{14}C]\)lactose from the proteoliposomes is followed (Fig. 1 inset). B, exit of \([^{14}C]\)lactose from proteoliposomes preloaded with 2.5 mM \([^{14}C]\)lactose under conditions where no sugar (●), 1 mM lactose (■), 1 mM fucose (▲), 1 mM β-PG (□), or 1 mM α-NG (▲) is present externally.

The rate of exit of \([^{14}C]\)lactose thus increases, compared with efflux, if a counter substrate is present externally, e.g. 1 mM fucose or 1 mM β-PG (Fig. 1B). From these data we conclude that fucose and β-PG are transported but, under the conditions employed, at a lower rate than lactose, which may reflect either a higher \(K_m\) and/or a lower \(V_{max}\) for these galactosides. When a sugar is bound tightly but not, or only very slowly, transported, one observes an inhibition of the rate of \([^{14}C]\)lactose efflux, as was observed for α-NG (Fig. 1B).

Fig. 2. The apparent affinity constants for lactose at the cytoplasmic and extracellular binding site of LacS. A, the initial rate of \([^{14}C]\)lactose exit from proteoliposomes is plotted as a function of the internal \([^{14}C]\)lactose concentration. The \([^{14}C]\)lactose exit rates were measured in the presence (●) and absence (■) of 1 mM unlabeled lactose outside the proteoliposomes (approximately four times the \(K_i^{app}\) at the cytoplasmic binding site). The inset shows the Lineweaver-Burk plots of the data. B, the initial rate of \([^{14}C]\)lactose uptake into proteoliposomes is plotted as a function of the external \([^{14}C]\)lactose concentration.

For several substrates \(IC_{50}\) values, which represent the concentration at which a solute inhibits the transport rate of...
The Binding Site Interacts Specifically with Galactose—From the data above it is clear that the galactose moiety of lactose is the critical determinant of LacS specificity and that no significant role of the C-1–OH is to be expected. D-galactose is distinct from other D-aldohexoses by the spatial orientation of the C-2, C-3, C-4, and C-6 hydroxyl groups to the pyranose ring structure. The spatial orientation of these hydroxyl groups is therefore expected to be important for substrate recognition. The C-2, C-3, and C-4 epimers of D-galactose (D-talose, D-gulose, and D-glucose) and galactosides with methoxy substitutions of the C-2 or C-6 hydroxy group impair the interaction with the cytoplasmic binding site (Table III), as no significant inhibition of [14C]lactose uptake is observed in the presence of these substrates.

4-O-Methyl-lactose, 2-deoxygalactose, and 6-deoxygalactose are bound and transported, albeit with lower transport rates.

The Cytoplasmic and Extracellular Binding Site Interact Differently with Galactose—IC50 values at the cytoplasmic and extracellular binding site were determined for galactosides that lack a hydroxyl group (Table IV). Compared with galactose the IC50 value at the cytoplasmic binding site is about 150-fold higher for 2-deoxygalactose and about 20-fold higher for 6-deoxygalactose. The IC50 values at the extracellular binding site, on the other hand, are about the same for galactose, 2-deoxygalactose, and 6-deoxygalactose. The C-2 and C-6 hydroxyl groups are thus important for binding at the cytoplasmic binding site but not for the interaction with the extracellular binding site. Substitution of the C-4 hydroxy group for a methoxy group did not impair the interaction with either binding site, as concluded from a comparison of the IC50 values for lactose and methyl-4-O-lactose (Table IV).

DISCUSSION

To dissect the interactions between substrates and the cytoplasmic and extracellular binding site of LacS, we made use of...
purified and unidirectionally reconstituted LacS and measured the transport and binding by LacS of a range of galactosides. We show that the LacS protein is specific for the galactose moiety and not for the galactose C-1 attached groups, e.g.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Presence and spatial orientation of hydroxyl or methoxy groups</th>
<th>Inhibition of $[^1]C_lactose$ uptake</th>
<th>Rate of heterogeneous exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>OH$^e$</td>
<td>90–95</td>
<td>100</td>
</tr>
<tr>
<td>Talose</td>
<td>OH$^e$</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>2-Deoxygalactose</td>
<td>OH$^e$</td>
<td>65 ± 5</td>
<td>28</td>
</tr>
<tr>
<td>Methyl-2-O-lactose</td>
<td>OH$^e$</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Glucose</td>
<td>OH$^e$</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Methyl-4-O-lactose</td>
<td>CH$_3$</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Fucose</td>
<td>OH$^e$</td>
<td>90–95</td>
<td>87</td>
</tr>
<tr>
<td>Methyl-6-O-galactose</td>
<td>OH$^e$</td>
<td>65 ± 5</td>
<td>48</td>
</tr>
</tbody>
</table>

a Differences compared to galactose are indicated in bold.

b See Footnote c in Table I.

c See Footnote d in Table I.

d n.s., not significant.

4-O/(2-O-Methyl-β-D-galactopyranosyl)-D-glucopyranoside, the presence and spatial orientation of the hydroxyl and methoxy groups on the galactose moiety are indicated.

Methyl-4-O-β-D-galactopyranosyl-β-D-glucopyranoside, the presence and spatial orientation of the hydroxyl and methoxy groups on the galactose moiety are indicated.

TABLE III

IC$_{50}$ values at the cytoplasmic and extracellular binding site of LacS for sugars lacking the C-2, C-4, or C-6 hydroxyl group

<table>
<thead>
<tr>
<th>Sugar</th>
<th>IC$<em>{50}$$</em>{cyt}$</th>
<th>IC$<em>{50}$$</em>{ext}$</th>
<th>IC$<em>{50}$$</em>{cyt}$/IC$<em>{50}$$</em>{ext}$</th>
<th>IC$<em>{50}$$</em>{cyt}$/IC$<em>{50}$$</em>{ext}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>1 (±0.5)</td>
<td>0.08 (±0.02)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2-Deoxygalactose</td>
<td>0.5 (±0.1)</td>
<td>12 (±1.5)</td>
<td>0.3–1.2</td>
<td>105–225</td>
</tr>
<tr>
<td>6-Deoxygalactose</td>
<td>1 (±0.5)</td>
<td>1.5 (±0.5)</td>
<td>0.3–3</td>
<td>10–33</td>
</tr>
<tr>
<td>Lactose</td>
<td>4 (±0.5)</td>
<td>0.29 (±0.05)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Methyl-4-O-lactose</td>
<td>6.5 (±1.5)</td>
<td>0.15 (±0.05)</td>
<td>0.9–2.2</td>
<td>0.3–0.7</td>
</tr>
</tbody>
</table>

a See Footnote b in Table II.

b See Footnote c in Table II.

c Methyl-4-O-β-D-galactopyranosyl-β-D-glucopyranoside.

TABLE IV

IC$_{50}$ values at the cytoplasmic and extracellular binding site of LacS for sugars lacking the C-2, C-4, or C-6 hydroxyl group

At the galactose C-4 position the binding site discriminates against the C-4 epimer of galactose (glucose), whereas 4-O-methyl-lactose with a methoxy group substituting the C-4 hydroxyl is as good a substrate as lactose (Table III). We cannot exclude the possibility that the C-4 hydroxyl is involved in accepting a hydrogen bond from the protein and thereby contributes to the specificity or affinity of substrate binding, because the C-4 methoxy groups may do so as well. The binding site also discriminates against the C-3 epimer of galactose, galactose (Table III). The importance of this position in sugar recognition by LacS could not be determined because galactosides modified at the C-3 position were not available.

Substrate selection based upon the ability of a sugar to fit into the binding site, rather than the ability to form specific H-bonds, was also suggested for GalP, as none of the hydroxyl groups seemed to be essential for transport (13). A similar conclusion can be drawn from the crystal structure of the allose-binding protein (6), which shows that the sugar ring is stacked between two parallel aromatic rings and a third perpendicular ring. As a result, binding of any hexose epimer other than galactose, is not recognized at the binding site. The IC$_{50}$ values at the cytoplasmic and extracellular binding site of LacS for sugars lacking the C-2, C-4, or C-6 hydroxyl group are even higher than for galactose (Table IV).
than the natural substrate d-allose and two deoxyalloses (C-3 and C-6) is sterically blocked. Similar to the interaction of 2-deoxygalactose and 6-deoxygalactose with LacS, the two deoxyalloses are expected to bind to the allose-binding protein with reduced affinity because of loss of a hydrogen bond. The above suggested substrate selection mechanism based on the ability of a sugar to fit into the binding site might thus reflect the fact that the spatial orientation of the hydroxyl groups determine which portion of the galactose molecule can participate in hydrophobic interactions with aromatic groups in the binding site of LacS.

In its normal conformation, the hydrogen atoms at C-3, C-4, C-5, and C-6 of galactose can be considered to form a hydrophobic plane as illustrated in Fig. 3A. The hydrophobic surface incorporating these hydrogen atoms would be a candidate for interaction with aromatic residues in the binding site. In contrast, bound [1-13C]galactose observed with cross-polarization magic-angle spinning NMR showed no significant difference in chemical shift compared with galactose in free solution, which is indicative of a comparable chemical environment (24). This would be consistent with C-1 being remote from the hydrophobic surface, as viewed in Fig. 3A, and existing in a more polar or well hydrated region of the binding site.

We showed that galactosides with large hydrophobic or hydrophilic groups at the galactose C-1(α) or C-1(β) position, e.g., ONPG, X-gal, raffinose, or methyl-3-O-β-d-galactopyranosyl-β-d-galactopyranoside, are transported with reasonable transport rates (Table 1). Also at the C-4 position some tolerance for substitutions was observed as methyl-4-O-lactose is transported as well as lactose (Tables III and IV). The binding site is apparently spacious in the areas surrounding the galactose C-4 and even more so at C-1.

Galactosides with hydrophobic groups attached to the C-1 position have decreased IC50 values at the cytoplasmic and extracellular binding site compared with galactose (Table II). The transport rates, however, are lower than those of galactose. α-NG, a strong inhibitor with an IC50 value of 4 μM compared with 80 μΜ for galactose, even inhibits efflux of [14C]lactose from proteoliposomes (Fig. 1B). The decreased transport can be explained thermodynamically by suggesting that the hydrophobic groups interact favorably with hydrophobic parts of the binding site, thereby decreasing the free energy of the sugar-transporter complex and thus increasing the activation energy for the reorientation of the binding sites.

The most important findings of this study concern the different interactions of the hydroxyl groups on the galactose moiety of galactosides with the cytoplasmic and extracellular binding site. Compared with galactose the IC50 values for 2-deoxygalactose and 6-deoxygalactose at the cytoplasmic binding site are about 150- and 20-fold increased, respectively, whereas they are unaltered at the extracellular binding site. We speculate that the C-2-OH and C-6-OH contribute highly to the affinity for galactose at the cytoplasmic binding site by forming hydrogen bonds with the protein, which does not take place when galactose is bound at the extracellular binding site (Table IV).

Differences in architecture of the cytoplasmic and extracellular binding site surrounding the substrate have also been reported for Glut 1 and GalP (4, 13). In these cases the differences do not represent differences in interactions of the binding sites with the hydroxyl groups, but rather differences in interactions with bulky substituents.

In conclusion, the observations that bulky substituents are only tolerated at the galactose C-1 and the C-4 positions and that the C-2 and C-6 hydroxyl groups contribute highly to the affinity at the cytoplasmic binding site suggest that the binding site and translocation pathway are spacious along the galactose C-1 to C-4 axes and restricted along the C-2 to C-6 axes. Fig. 3B shows the C-1 to C-4 axes and C-2 to C-6 axes in methyl-4-O-lactose. Given these interactions and the structures of the galactosides (even trisaccharides) transported, it seems reasonable to suggest that the sugars move through the protein along their galactose C-1 to C-4 axes.

REFERENCES

Specificity of Lactose Transport