

## 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 for bulkier groups is not tolerated at these positions. Large hydrophobic or hydrophilic substitutions on the galactose C-1  $\alpha$  or  $\beta$  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  $IC_{50}$  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 hexuronides (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).<sup>1</sup>

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  $\beta$ -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<sup>+</sup> 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), the human intestinal brush border glucose/Na<sup>+</sup> cotransporter SGLT1 (17), the *Trypanosoma brucei* bloodstream form transporter THT1 (18), and the human active renal hexose transporter (19). Most of

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<sup>1</sup> 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**—D-(glucose-1-<sup>14</sup>C)lactose (2.11 teslabecquerel/mol) was obtained from the Radiochemical Center, Amersham Pharmacia Biotech. Ni-nitrilotriacetic 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 L- $\alpha$ -phosphatidylcholine were obtained from Avanti Polar Lipids and Sigma, respectively. 4-*O*- $\beta$ -D-Galactopyranosyl-D-glucose (lactose), 6-*O*- $\alpha$ -D-galactopyranosyl-D-glucose (melibiose), *O*- $\alpha$ -D-galactopyranosyl-(1,6)- $\alpha$ -D-galactopyranosyl-(1,6)- $\alpha$ -D-glucopyranosyl-(1,2)- $\beta$ -D-fructofuranoside (stachyose), *O*- $\alpha$ -D-galactopyranosyl-(1,6)- $\alpha$ -D-glucopyranosyl-(1,2)- $\beta$ -D-fructofuranoside (raffinose),  $\alpha$ -D-talose,  $\alpha$ -naphthyl  $\alpha$ -D-galactopyranoside ( $\alpha$ -NG),<sup>2</sup>  $\alpha$ -naphthyl  $\beta$ -D-galactopyranoside ( $\beta$ -NG), 2-deoxy-D-galactose, D-fucose, D-glucose, D-gulose, methyl-3-*O*- $\beta$ -D-galactopyranosyl- $\beta$ -D-galactopyranoside, methyl  $\beta$ -D-thiogalactoside (TMG), methyl-4-*O*- $\beta$ -D-galactopyranosyl- $\beta$ -D-glucopyranoside, 4-*O*-(2-*O*-methyl- $\beta$ -D-galactopyranosyl)-D-glucopyranoside, 6-*O*-methyl-D-galactopyranoside, *O*-nitrophenyl  $\beta$ -D-galactopyranoside ( $\beta$ -ONPG), *O*-nitrophenyl- $\alpha$ -D-galactopyranoside ( $\alpha$ -ONPG), phenyl- $\beta$ -D-galactoside ( $\beta$ -PG), *S*- $\beta$ -D-galactopyranosyl-(1,1)- $\beta$ -D-galactopyranoside (TDG), and thio- $\beta$ -D-galactopyranoside were obtained from Sigma. Isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside (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 ST11 $\Delta$ lacS expressing his-tagged LacS from pGKhis was grown semi-aerobic at 42 °C in (B)elliker broth (22) supplemented with 0.5% beef extract, 0.5% lactose, and 4  $\mu$ g/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 performed as 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 MgSO<sub>4</sub>) 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  $\times g$ , 15 °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), aliquots of 1  $\mu$ l of proteoliposome suspension were diluted into 200  $\mu$ l of KPM containing different concentrations of [<sup>14</sup>C]lactose. The uptake of [<sup>14</sup>C]lactose was stopped at different time intervals by dilution of the sample with 2 ml of ice-cold 0.1 M LiCl and rapid filtering on 0.45- $\mu$ m cellulose nitrate filters (Schleicher & Schuell). To calculate the actual external lactose concentration, we accounted for the lactose brought into the external buffer upon the addition of the proteoliposome suspension. IC<sub>50</sub> values were determined by measuring the uptake of [<sup>14</sup>C]lactose present at 50  $\mu$ M (approximately 0.2 times the  $K_m^{(app)}$  at the cytoplasmic binding site) in the presence of at least six different concentrations of competing sugars. The proteoliposomes were preloaded with 10 mM lactose (approximately two times the  $K_m^{(app)}$  at the extracellular binding site).

<sup>2</sup> The abbreviations used are:  $\alpha$ -NG and  $\beta$ -NG,  $\alpha$ -naphthyl  $\alpha$ -D-galactopyranoside and  $\alpha$ -naphthyl  $\beta$ -D-galactopyranoside;  $\beta$ -ONPG and  $\alpha$ -ONPG, *O*-nitrophenyl- $\beta$ -D-galactopyranoside and *O*-nitrophenyl- $\alpha$ -D-galactopyranoside;  $\beta$ -PG, phenyl- $\beta$ -D-galactoside; X-gal, 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside; TDG, *S*- $\beta$ -D-galactopyranosyl-(1,1)- $\beta$ -D-galactopyranoside; TMG, methyl  $\beta$ -D-thiogalactoside; IPTG, isopropyl-1-thio- $\beta$ -D-galactopyranoside.

**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 [<sup>14</sup>C]lactose. At time point zero, 1  $\mu$ l of proteoliposome suspension was diluted into 1 ml of KPM without (efflux) or with (exchange) 1 mM lactose (approximately four times the  $K_m^{(app)}$  at the cytoplasmic binding site), and the exit of lactose was stopped at different time points as described above. IC<sub>50</sub> values at the extracellular binding site were measured using proteoliposomes that were preloaded with 1 mM of [<sup>14</sup>C]lactose (approximately 0.2 times the  $K_m^{(app)}$  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  $K_m^{(app)}$  at the cytoplasmic binding site). The exit of [<sup>14</sup>C]lactose was stopped at different time points as described above. To determine whether or not galactose analogs accelerate the exit of [<sup>14</sup>C]lactose and are thus transported, proteoliposomes were preloaded with 2.5 mM [<sup>14</sup>C]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 [<sup>14</sup>C]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 [<sup>14</sup>C]lactose retained inside the proteoliposomes after complete equilibration of [<sup>14</sup>C]lactose-preloaded proteoliposomes with external buffer is not significant compared with the background binding and was thus not corrected for.

The uptake of [<sup>14</sup>C]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  $Y$  (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 mM) with the specific internal volume of the proteoliposomes. Initial rates were calculated from the amount of lactose inside the proteoliposomes at time point zero ( $A$ ) multiplied with the decay constant ( $B$ ).

The specific internal volume of the [<sup>14</sup>C]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  $\mu$ l/mg lipid). The amount of radioactive label at time point zero was determined by extrapolation.

IC<sub>50</sub> values were determined from the inhibition curves that were fitted with a logistic function,

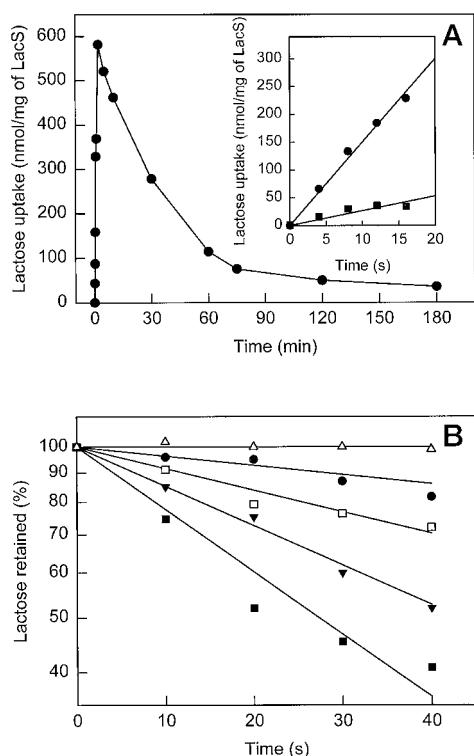
$$V = (V_{100} - V_0) / [1 + (I/IC_{50})] + V_0 \quad (\text{Eq. 2})$$

where  $V_{100}$  and  $V_0$  correspond to the rate of uptake in the absence of inhibitor and the rate of uptake at infinite inhibition, respectively;  $I$  is the concentration of inhibitor, and IC<sub>50</sub> is the concentration at which the inhibitor inhibits the uptake 50%.

**Miscellaneous**—Protein determinations on membrane vesicles were performed with the Bio-Rad DC protein assay according to the manufacturer's instructions (Bio-Rad). The concentration LacS in the elution fraction after Ni-nitrilotriacetic acid purification was determined spectrophotometrically at 280 nm ( $\epsilon_{280} = 1.08 \text{ (mg/ml)}^{-1} \text{ cm}^{-1}$ ). As Triton X-100 absorbs at 280 nm, corrections were made for the contribution of (i) free detergent to the  $A_{280}$  by subtracting the  $A_{280}$  of the elution buffer and (ii) Triton X-100 molecules bound to LacS. The amount of Triton X-100 bound to LacS was estimated from the  $A_{280}/A_{290}$  ratio of the sample and using the  $A_{280}/A_{290}$  ratios of a Triton X-100 solution and that of LacS protein in dodecyl-maltoside. Three-dimensional molecular modeling of the substrates was done using Hyperchem Lite, Hypercube, Inc. Scientific Software.

#### RESULTS

**Substrate Binding and Transport by LacS**—To dissect the interactions between substrates and the cytoplasmic or extra-

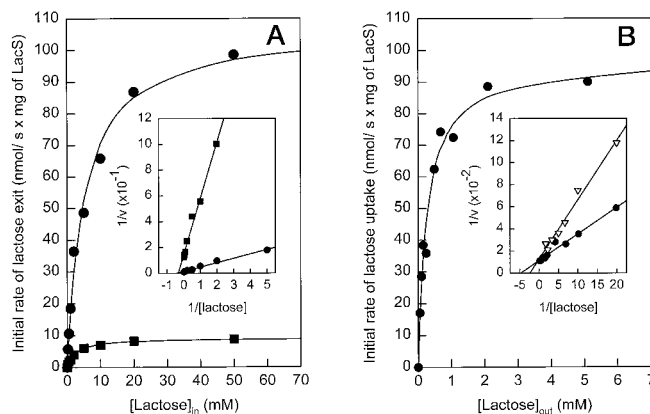


**FIG. 1. Counterflow, exchange, and efflux catalyzed by LacS.** A, LacS-mediated lactose transport assayed in a counterflow assay. The reaction was started by diluting proteoliposomes equilibrated with 10 mM lactose 200 times into buffer with 3.6  $\mu\text{M}$  [ $^{14}\text{C}$ ]lactose. The [ $^{14}\text{C}$ ]lactose uptake over the first 20 s in the absence (●) and presence (■) of a 20-fold excess of galactose is shown in the inset. B, exit of [ $^{14}\text{C}$ ]lactose from proteoliposomes preloaded with 2.5 mM [ $^{14}\text{C}$ ]lactose under conditions where no sugar (●), 1 mM lactose (■), 1 mM fucose (▼), 1 mM  $\beta$ -PG (□), or 1 mM  $\alpha$ -NG ( $\Delta$ ) is present externally.

cellular binding site, the LacS protein was purified and unidirectionally reconstituted. The membrane reconstitution protocol yields proteoliposomes in which the LacS protein is inserted inside-out into the membrane, implying that the extracellular binding site is at the inner surface of the proteoliposomes and the cytoplasmic binding site is facing the outside of the proteoliposomes.

Transport and binding of several galactosides were monitored in three types of assays, that is counterflow, exchange, and efflux down the concentration gradient. In the counterflow and the exchange transport assays, two pools of differently labeled galactosides, one inside and one outside the proteoliposomes, equilibrate in time through carrier-mediated transport. In the counterflow assay [ $^{14}\text{C}$ ]lactose is present outside the proteoliposomes, whereas in the exchange and efflux assays [ $^{14}\text{C}$ ]lactose is present inside the proteoliposomes. In the efflux assay exit of [ $^{14}\text{C}$ ]lactose from the proteoliposomes is followed in the absence of external substrate.

Uptake of [ $^{14}\text{C}$ ]lactose in the counterflow assay can initially be approximated with a linear function (Fig. 1A, inset). Eventually, the [ $^{14}\text{C}$ ]lactose redistributes until the external and internal concentrations have become equal (Fig. 1A). Inhibition of the initial rate of uptake of [ $^{14}\text{C}$ ]lactose as a consequence of the presence of a 20-fold excess of a nonlabeled sugar, e.g. galactose (Fig. 1A, inset) outside the proteoliposomes, implies that the sugar is bound and/or transported by LacS. To establish that the inhibitor is indeed transported, we used acceleration of [ $^{14}\text{C}$ ]lactose exit as criterion. Exit down the concentration gradient of [ $^{14}\text{C}$ ]lactose from proteoliposomes is more than 10 times slower when sugar is absent than when saturating amounts of unlabeled lactose are present externally (Fig. 1B).



**FIG. 2. The apparent affinity constants for lactose at the cytoplasmic and extracellular binding site of LacS.** A, the initial rate of [ $^{14}\text{C}$ ]lactose exit from proteoliposomes is plotted as a function of the internal [ $^{14}\text{C}$ ]lactose concentration. The [ $^{14}\text{C}$ ]lactose exit rates were measured in the presence (●) and absence (■) of 1 mM unlabeled lactose outside the proteoliposomes (approximately four times the  $K_m^{\text{app}}$  at the cytoplasmic binding site). The inset shows the Lineweaver-Burk plots of the data. B, the initial rate of [ $^{14}\text{C}$ ]lactose uptake into proteoliposomes is plotted as a function of the external [ $^{14}\text{C}$ ]lactose concentration. The uptake rates were measured in the presence of 10 mM unlabeled lactose inside the proteoliposomes (approximately two times the  $K_m^{\text{app}}$  at the extracellular binding site). The inset shows the Lineweaver-Burk plots of the initial rates of [ $^{14}\text{C}$ ]lactose uptake into proteoliposomes as a function of the external [ $^{14}\text{C}$ ]lactose concentration in the presence (▼) and absence (●) of 80  $\mu\text{M}$  galactose externally. Michaelis-Menten analysis of the data in A and B yielded a  $K_m^{\text{app}}$  for lactose at the extracellular binding site of  $5 \pm 0.5$  mM and at the cytoplasmic binding site of  $0.25 \pm 0.05$  mM; the apparent  $K_i$  for galactose at the cytoplasmic binding site was 80  $\mu\text{M}$ .

The rate of exit of [ $^{14}\text{C}$ ]lactose thus increases, compared with efflux, if a counter substrate is present externally, e.g. 1 mM fucose or 1 mM  $\beta$ -PG (Fig. 1B). From these data we conclude that fucose and  $\beta$ -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  $V_{\text{max}}$  for these galactosides. When a sugar is bound tightly but not, or only very slowly, transported, one observes an inhibition of the rate of [ $^{14}\text{C}$ ]lactose efflux, as was observed for  $\alpha$ -NG (Fig. 1B).

**Apparent Affinities and  $IC_{50}$  Values at the Cytoplasmic and Extracellular Binding Site**—In Fig. 2A the initial rates of LacS-mediated, exit of [ $^{14}\text{C}$ ]lactose from proteoliposomes preloaded with different concentrations of [ $^{14}\text{C}$ ]lactose, are plotted as a function of the internal [ $^{14}\text{C}$ ]lactose concentration. In the exchange transport assays (closed circles), the cytoplasmic binding site faces a fixed near saturating amount of unlabeled lactose (1 mM, approximately four times the  $K_m^{\text{app}}$  at the cytoplasmic binding site). The apparent affinity constant of lactose at the extracellular binding site, which reflect a transport constant rather than a binding constant, was  $5 \pm 0.5$  mM. In the efflux experiment (closed squares), the cytoplasmic binding site faces maximally 50  $\mu\text{M}$  lactose, present as a result of the dilution of the proteoliposomes into the buffer. The apparent affinity constant at the extracellular binding site estimated from the efflux assay was comparable to that of the exchange reaction, but the  $V_{\text{max}}$  was more than ten times lower. Fig. 2B depicts the uptake of [ $^{14}\text{C}$ ]lactose as a function of the external lactose concentration. The proteoliposomes were preloaded with 10 mM lactose so that the extracellular binding site faces near saturating amounts of lactose (approximately two times the  $K_m^{\text{app}}$  at the extracellular binding site). The apparent affinity constant of lactose at the cytoplasmic binding site was determined to be  $0.25 \pm 0.05$  mM.

For several substrates  $IC_{50}$  values, which represent the concentration at which a solute inhibits the transport rate of

TABLE I

Binding and transport of galactose and C-1-substituted galactosides

Sugar <sup>a</sup>	Inhibition of [ <sup>14</sup> C]lactose uptake <sup>b</sup>	Rate of heterologous exchange <sup>c</sup>
	%	%
Galactose	90–95	100
Lactose (galp-(β,1-O-4)-glucp)	90–95	96
TDG (galp-(β,1-S-1)-βgalp)	90–95	66
Melibiose (galp-(α,1-O-6)-glucp)	90–95	57
Methyl-3-O-β-D-galp-β-D-galp	27 ± 5	36
Raffinose (galp-(α,1-O-6)-glucp-(α,1-O-2)-frucf)	72 ± 5	28
Stachyose (galp-(α,1-O-6)-galp-(α,1-O-6)-glucp-(α,1-O-2)-frucf)	n.s. <sup>d</sup>	2
1-Thiogalactose (galp-β-SH)	90–95	113
TMG (galp-β-S-methyl)	90–95	66
IPTG (galp-β-S-isopropyl)	>95	6
β-PG (galp-β-O-phenyl)	>95	28
α-ONPG (galp-α-O-nitrophenyl)	>95	32
β-ONPG (galp-β-O-nitrophenyl)	90–95	19
α-NG (galp-α-O-naphthyl)	>95	-12
β-NG (galp-β-O-naphthyl)	>95	6
X-gal (galp-β-O-(5-Br-4-Cl-3-indolyl))	>95	32

<sup>a</sup> Linkages and C-1 attached groups are depicted for convenience; for full names see "Materials and Methods."

<sup>b</sup> Inhibition of the initial rate of [<sup>14</sup>C]lactose uptake was measured in the presence of a 20-fold excess of the depicted sugar over [<sup>14</sup>C]lactose. The counterflow assay was done with proteoliposomes preloaded with 10 mM lactose and an external [<sup>14</sup>C]lactose concentration of 50 μM.

<sup>c</sup> Rate of [<sup>14</sup>C]lactose exit from proteoliposomes when 5 mM of the depicted sugar was present externally. The internal concentration of [<sup>14</sup>C]lactose was 2.5 mM. Rates are relative to [<sup>14</sup>C]lactose/galactose exchange, which was taken as 100%. [<sup>14</sup>C]lactose efflux was taken as 0%. Rates larger than 0% indicate that the depicted sugar is transported. Transport rates between 0 and 15% have an error range of ±3%, between 15 and 40% ± 6%, and between 40 and 100% ± 9%.

<sup>d</sup> n.s., not significant.

[<sup>14</sup>C]lactose for 50%, were determined at the cytoplasmic and extracellular binding site. Inhibition of lactose uptake by the presence of external galactose is competitive as shown in the inset of Fig. 2B; the apparent  $K_I$  for galactose at the cytoplasmic binding site was 80 μM.

**The Binding Site and Translocation Pathway Allow Accommodation of Large Groups on the Galactose C-1**—Table I summarizes the data on the inhibition of lactose uptake (column 2) and acceleration of lactose exit (column 3) by galactosides with a variety of hydrophobic and hydrophilic groups linked to the galactose C-1(α) or C-1(β). The exchange rates are depicted as percentage relative to that of galactose. The data reflect interaction of the sugars with the cytoplasmic binding site and the subsequent transport to the inside of the proteoliposomes. Overall, the experiments show that the LacS protein can accommodate large hydrophobic or hydrophilic groups at the galactose C-1, as substrates like α-ONPG, X-gal, raffinose, or methyl-3-O-β-D-galactopyranosyl-β-D-galactopyranoside are transported with reasonable transport rates.

**Hydrophobic Groups Increase Binding Affinity but Decrease Transport Rates**—Large hydrophobic groups attached to the C-1 position of galactose yielded  $IC_{50}$  values for the cytoplasmic and extracellular binding site that were much lower than that of galactose (Table II). The sugars, however, are transported with lower rates than galactose, and in the case of α-NG a significant inhibition of the efflux was even observed (Fig. 1B).  $IC_{50}$  values at the extracellular binding site were only determined for isopropyl-1-thio-β-D-galactopyranoside (IPTG) as α-NG and α-ONPG readily diffuse out of the proteoliposomes, which would have led to an overestimation. The  $IC_{50}$  values for the hydrophobic galactosides are possibly somewhat influenced by the fact that these solutes partition to some extent in the membrane. The phenomenon that hydrophobic groups increase binding affinity but decrease transport rates is also clear from

TABLE II

Transport rates and  $IC_{50}$  values for galactose and galactosides with C-1 hydrophobic substitutions at the cytoplasmic and extracellular binding site of LacS

Sugar <sup>a</sup>	$IC_{50}^{ext\ b}$	$IC_{50}^{cyt\ c}$	Rate of heterologous exchange <sup>d</sup>
	mM	mM	%
Galactose	1 (±0.5)	0.08 (±0.02)	100
α-ONPG (galp-α-O-nitrophenyl)	n.d. <sup>e</sup>	<0.08 (±0.02)	32
IPTG (galp-β-S-isopropyl)	0.008 (±0.003)	0.016 (±0.007)	6
α-NG (galp-α-O-naphthyl)	n.d.	0.004 (±0.003)	-12

<sup>a</sup> For full names see "Materials and Methods."

<sup>b</sup>  $IC_{50}^{ext}$ , reflecting the extracellular binding site, was determined by measuring the exit of [<sup>14</sup>C]lactose from proteoliposomes at variable internal concentrations of inhibitor. The exit of [<sup>14</sup>C]lactose, present at a concentration of 1 mM, was measured in an exchange reaction with 0.5 mM lactose present externally.

<sup>c</sup>  $IC_{50}^{cyt}$ , reflecting the cytoplasmic binding site was determined by measuring the uptake of [<sup>14</sup>C]lactose into proteoliposomes at variable external concentrations of competing sugar. [<sup>14</sup>C]lactose, present at a concentration of 50 μM, was taken up in a counterflow reaction with 10 mM lactose inside the proteoliposomes.

<sup>d</sup> See Footnote c in Table I.

<sup>e</sup> n.d., not determined.

Table I. Here, the hydrophobic galactosides that are transported with intermediate transport rates (β-PG and α-ONPG) show equal or stronger inhibition of [<sup>14</sup>C]lactose uptake than galactose. The hydrophilic galactosides that are transported with intermediate transport rates (methyl-3-O-β-D-galactopyranosyl-β-D-galactopyranoside and raffinose) show only moderate inhibition of [<sup>14</sup>C]lactose uptake.

**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 hydroxyl impair the interaction with the cytoplasmic binding site (Table III), as no significant inhibition of [<sup>14</sup>C]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**— $IC_{50}$  values at the cytoplasmic and extracellular binding site were determined for galactosides that lack a hydroxyl group (Table IV). Compared with galactose the  $IC_{50}$  value at the cytoplasmic binding site is about 150-fold higher for 2-deoxygalactose and about 20-fold higher for 6-deoxygalactose. The  $IC_{50}$  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 hydroxyl group for a methoxy group did not impair the interaction with either binding site, as concluded from a comparison of the  $IC_{50}$  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

TABLE III  
The importance of the C-2-OH<sup>eq</sup>, C-3-OH<sup>eq</sup>, C-4-OH<sup>ax</sup>, and C-6-OH for substrate recognition

Sugar	Presence and spatial orientation of hydroxyl or methoxy groups <sup>a</sup>				Inhibition of [ <sup>14</sup> C]lactose uptake <sup>b</sup>	Rate of heterologous exchange <sup>c</sup>
	C-2	C-3	C-4	C-6		
Galactose	OH <sup>eq</sup>	OH <sup>eq</sup>	OH <sup>ax</sup>	OH	90–95	100
Talose	<b>OH<sup>ax</sup></b>	OH <sup>eq</sup>	OH <sup>ax</sup>	OH	n.s. <sup>d</sup>	n.s.
2-Deoxygalactose		OH <sup>eq</sup>	OH <sup>ax</sup>	OH	68 ± 5	28
Methyl-2-O-lactose <sup>e</sup>	<b>CH<sub>3</sub><sup>eq</sup></b>	OH <sup>eq</sup>	OH <sup>ax</sup>	OH	n.s.	n.s.
Gulose	OH <sup>eq</sup>	<b>OH<sup>ax</sup></b>	OH <sup>ax</sup>	OH	n.s.	n.s.
Glucose	OH <sup>eq</sup>	OH <sup>eq</sup>	<b>OH<sup>eq</sup></b>	OH	n.s.	n.s.
Methyl-4-O-lactose <sup>f</sup>	OH <sup>eq</sup>	OH <sup>eq</sup>	<b>CH<sub>3</sub><sup>ax</sup></b>	OH	90–95	87
Fucose	OH <sup>eq</sup>	OH <sup>eq</sup>	OH <sup>ax</sup>		42 ± 5	48
Methyl-6-O-galactose	OH <sup>eq</sup>	OH <sup>eq</sup>	OH <sup>ax</sup>	<b>CH<sub>3</sub></b>	n.s.	n.s.

<sup>a</sup> Differences compared to galactose are indicated in bold.

<sup>b</sup> See Footnote c in Table I.

<sup>c</sup> See Footnote d in Table I.

<sup>d</sup> n.s., not significant.

<sup>e</sup> 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.

<sup>f</sup> 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 IV  
IC<sub>50</sub> values at the cytoplasmic and extracellular binding site of LacS for sugars lacking the C-2, C-4, or C-6 hydroxyl group

Sugar	IC <sub>50</sub> <sup>ext a</sup>	IC <sub>50</sub> <sup>cyt b</sup>	IC <sub>50</sub> <sup>ext</sup> /IC <sub>50</sub> <sup>ext</sup> <sub>galactose</sub>	IC <sub>50</sub> <sup>cyt</sup> /IC <sub>50</sub> <sup>cyt</sup> <sub>galactose</sub>
	<i>mM</i>	<i>mM</i>		
Galactose	1 (±0.5)	0.08 (±0.02)	1	1
2-Deoxygalactose	0.5 (±0.1)	12 (±1.5)	0.3–1.2	105–225
6-Deoxygalactose	1 (±0.5)	1.5 (±0.5)	0.3–3	10–33
			IC <sub>50</sub> <sup>ext</sup> /IC <sub>50</sub> <sup>ext</sup> <sub>lactose</sub>	IC <sub>50</sub> <sup>cyt</sup> /IC <sub>50</sub> <sup>cyt</sup> <sub>lactose</sub>
Lactose	4 (±0.5)	0.29 (±0.05)	1	1
Methyl-4-O-lactose <sup>c</sup>	6.5 (±1.5)	0.15 (±0.05)	0.9–2.2	0.3–0.7

<sup>a</sup> See Footnote b in Table II.

<sup>b</sup> See Footnote c in Table II.

<sup>c</sup> Methyl-4-O-β-D-galactopyranosyl-β-D-glucopyranoside.

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. the glucose moiety in the case of lactose (Table I and III). It is interesting to note that glucose, the sugar moiety of lactose that serves as a carbon and an energy source in *S. thermophilus*, is not recognized at the binding site. The IC<sub>50</sub> values for lactose at both binding sites are even higher than for galactose (Table IV). The observations that the affinity for galactose (and lactose) at the cytoplasmic binding site is 20-fold higher than at the extracellular binding site (Fig. 2) and that galactose is preferred over lactose at the cytoplasmic binding site (Table IV) are consistent with the view that LacS is designed to catalyze an efficient lactose/galactose exchange, rather than a one directional inward sugar flux.

Galactose is distinct from other aldohexoses by the spatial orientation of the hydroxyl and hydrogen groups on the pyranose ring, which is expected to play a role in the recognition of the substrate by the protein. Specificity of the LacS protein for galactose can be based upon the formation of hydrogen bonds between the C-2, C-3, C-4, and/or C-6 hydroxyl groups and specific groups in the binding site but could also be merely based upon the ability of a sugar to fit into the binding site. The latter possibility is suggested by the experiments on the interaction of LacS with the C-2 and C-6 position of galactose. 2-Deoxygalactose and 6-deoxygalactose are bound and transported by LacS with reasonable rates, whereas the C-2-epimer of galactose (talose), methyl-2-O-lactose, and methyl-6-O-galactose are not (Table III). The impaired interaction of the binding site with talose, methyl-2-O-lactose, and methyl-6-O-galactose is therefore not caused by the lack of an essential hydrogen

bond but rather by an impaired fit into the binding site. The hydroxyl group at the galactose C-1 also does not form a hydrogen bond that contributes to the specificity, as C-1-substituted galactosides that are not able to form a hydrogen bond are transported (Table I). The lack of specificity for the α- or β-anomer of galactose was also shown by cross-polarization magic-angle spinning NMR (24). Although hydrogen bonding with the galactose C-1, C-2, and C-6 hydroxyl groups is not essential for the specificity of LacS for galactose, these interactions can contribute to the affinity for galactose binding. Indeed, 2-deoxygalactose, 6-deoxygalactose, and for instance, lactose have larger IC<sub>50</sub> values than galactose (Table IV).

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, gulose (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

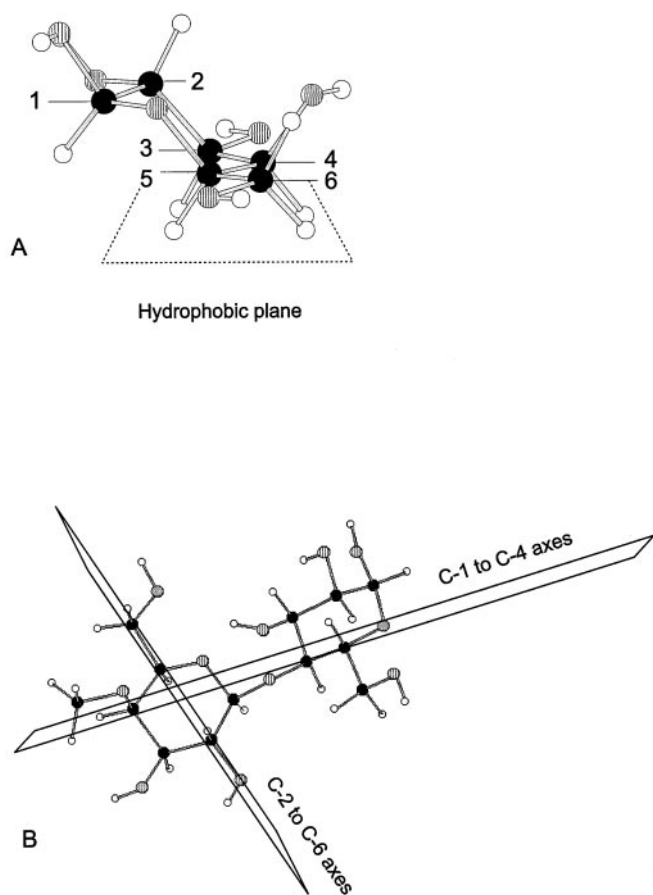


FIG. 3. Structures of  $\beta$ -D-galactose and methyl-4-O-lactose. *A*,  $\beta$ -D-galactose viewed along the C-6 to C-3 axes showing that the C-3, C-4, C-5, and C-6 hydrogen atoms can form a hydrophobic plane. *B*, methyl-4-O- $\beta$ -D-galactopyranosyl- $\beta$ -D-glucopyranoside. Shown are the C-1 to C-4 axes on the galactose moiety, which is where LacS tolerates substitution of the hydroxyl groups for larger groups, and the C-2 to C-6 axes, which provide high affinity binding at the cytoplasmic binding site. Carbon, oxygen, and hydrogen atoms are in black, stripes, and white, respectively.

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- $^{13}$ C]D-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( $\alpha$ ) or C-1( $\beta$ ) position, *e.g.*

ONPG, X-gal, raffinose, or methyl-3-O- $\beta$ -D-galactopyranosyl- $\beta$ -D-galactopyranoside, are transported with reasonable transport rates (Table I). 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  $IC_{50}$  values at the cytoplasmic and extracellular binding site compared with galactose (Table II). The transport rates, however, are lower than those of galactose.  $\alpha$ -NG, a strong inhibitor with an  $IC_{50}$  value of 4  $\mu$ M compared with 80  $\mu$ M for galactose, even inhibits efflux of [ $^{14}$ C]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  $IC_{50}$  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.

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