A Nontransportable Substrate for Lactose Permease

Christoph Seibert, Wolfgang Dörner, and Fritz Jähnig*

Max-Planck-Institut für Biologie, Abteilung Membranbiochemie, Correnssstrasse 38, D-72076 Tübingen, Germany

Received February 14, 1995; Revised Manuscript Received April 10, 1995[⊗]

ABSTRACT: A substrate for lactose permease of *Escherichia coli* was synthesized that binds to the protein with a relatively high affinity, but is not transported to any detectable extent. This substrate, 6′-[(*N*-phenylalanylphenylalanyl)amino]hexyl 1-thio-β-D-galactoside, is a peptide galactoside composed of a bulky aromatic dipeptide that is linked to galactose via an aminohexyl spacer. Binding of the peptide galactoside to lactose permease in cytoplasmic membranes was determined in a competition assay yielding a dissociation constant of 150 μM. Transport was measured by a counterflow assay using lipid vesicles with reconstituted lactose permease. An upper limit for the rate constant of transport was obtained as 0.02 s^{-1}, 3 orders of magnitude smaller than the value for lactose.

Lactose permease (LP)[1] is an integral protein of the cytoplasmic membrane of *Escherichia coli* that catalyzes the symport of a galactoside and a proton across the membrane (for rewiews, see Kaback, 1986; Wright et al., 1986). Lactose permease is one of the best characterized transport proteins, but as for any other transport protein, its three-dimensional structure remains unknown. The rules to crystallize transport proteins are not known, but one may expect that the protein must be immobilized in one conformation. The simplest way to achieve this may be the binding of a substrate which fixes the protein in one conformation. Such a strategy was at least successful for the crystallization of a number of sugar binding proteins (Quiocho & Vyas, 1984). This would imply that the substrate binds to LP with high affinity, but is not transported. Such a substrate would, furthermore, permit us to perform so-called recruiting experiments which provide information not accessible from transport measurements. For the glucose carrier of erythrocytes, such a substrate is available (Appleman & Lienhard, 1985). The present paper describes a nontransportable substrate for LP.

LP binds a large variety of α- and β-galactosides, but all of them are transported. Substrate recognition seems to require a galactopyranosyl ring in the D-configuration with a free 6-hydroxyl group. The aglycon may vary considerably, but nonetheless contributes to the affinity (Sandermann, 1977). Especially aromatic aglycons lead to a high affinity, examples being *p*-nitrophenyl α-D-galactoside (NpαGal) with a nitrophenyl group and a dissociation constant K_d = 22 μM as well as 6′-[(*N*-dansyl)amino]hexyl-1-thio-β-D-galactoside (DnsHxSGal) with a dansyl group and K_d = 5 μM (Schuldiner et al., 1974; Schuldiner & Kaback, 1977; Wright et al., 1981). In contrast, carbohydrate aglycons usually lead to a low affinity (lactose, for example, has a K_d = 14 mM), the only known exception being β-d-galactosyl 1-thio-β-D-galactoside (GalSGal) with a K_d = 70 μM. It has been proposed that the galactoside binding site consists of two different subsites, one for the galactosyl ring and one for the hydrophobic aromatic or carbohydrate aglycons (Wright et al., 1981).

A strategy for the synthesis of a nontransportable substrate, therefore, could be the use of a galactosyl group for recognition of the specific subsite and a bulky hydrophobic aglycons, which enables interaction with the unspecific subsite and prohibits transport. In this study, we demonstrate that such a strategy is indeed successful. If two phenylalanine residues and a hexyl chain linking it to the galactoside are chosen for the hydrophobic part, the substrate binds, but is not transported.

MATERIALS AND METHODS

Materials. Dicyclohexylcarbodiimide and *N*-hydroxysuccinimide were purchased from Merck (Darmstadt). [(9-Fluorenylmethoxy)carbonyl]-L-phenylalanine was from Novabiochem (Heidelberg). β-D-Galactosyl 1-thio-β-D-galactoside (GalSGal) and gramicidin D were from Sigma (München), and valinomycin was from Serva (Heidelberg). β-D-[6-^{3}H]-Galactosyl 1-thio-β-D-[6′-^{3}H]galactoside ([6,6′-$^3H_2$]GalSGal) was obtained from CEA (Gif-sur-Yvette), and *p*-nitrophenyl α-D-[6-^{3}H]galactoside ([6-^{3}H]NpαGal) was a gift of P. Overath.

6′-Aminohexyl 1-thio-β-D-galactoside was synthesized as described (Chipowsky & Lee, 1973). [(9-Fluorenylmethoxy)-carbonyl]-L-phenylalanyl-L-phenylalanine was prepared from [(9-fluorenylmethoxy)carbonyl]-L-phenylalanine and L-phenylalanine by the *N*-hydroxysuccinimide ester method (Anderson et al., 1964).

Thin-layer chromatography was carried out on precoated silica gel-60 F_{254} plates (Merck, Darmstadt) in the solvent chloroform/methanol/acetic acid/water (65:25:3:3 v/v). Compounds were visualized by one or more of the following procedures: fluorescence at 254 nm, ninhydrin, a modified chlorine reagent (Barrolier, 1961), and orcine reagent.

Analytical RP-HPLC was performed on a Nucleosil 300 C18 column (particle size 5 μm, 250 × 2 mm) (Grom, Herrenberg) at a flow rate of 0.3 mL/min using System Gold equipment (Beckman, Scan Ramon). Water containing

[⊗] Abstract published in *Advance ACS Abstracts,* June 1, 1995.

[1] Abbreviations: LP, lactose permease; CPM vesicles, cytoplasmic membrane vesicles; Phe_2HxSGal, 6′-[(*N*-phenylalanylphenylalanyl)-amino]hexyl 1-thio-β-D-galactoside; GalSGal, β-D-galactosyl 1-thio-β-D-galactoside; NpαGal, *p*-nitrophenyl α-D-galactoside; DnsHxSGal, 6′-[(*N*-dansyl)amino]hexyl 1-thio-β-D-galactoside; DTT, dithiothreitol; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; RP-HPLC, reverse-phase high-performance liquid chromatography; ES-MS, electrospray mass spectrometry.

0006-2960/95/0434-7819$09.00/0

0.12% TFA (solvent A) and acetonitrile containing 0.1% TFA (solvent B) were used as solvents, and a gradient from 10% (v/v) to 100% (v/v) B within 45 min was applied. The purity of the peptide galactoside was judged from the area of the peak at 210 nm. The identity of the peptide galactoside was proven by pneumatically assisted electrospray mass spectrometry on an API III triple quadrupole mass spectrometer equipped with an IonSpray source (Sciex, Thornhill, Ontario).

Synthesis of 6′-[[[(9-Fluorenylmethoxy)carbonyl]-L-phenylalanyl-L-phenylalanyl]amino]hexyl 1-Thio-β-D-galactoside. The *N*-hydroxysuccinimide ester of [(9-fluorenylmethoxy)-carbonyl]-L-phenylalanyl-L-phenylalanine was prepared according to the method of Anderson et al. (1964). A solution of 0.1 g (0.16 mmol) of this compound in 4 mL of tetrahydrofuran was added to a solution of 0.05 g (0.16 mmol) of 6′-aminohexyl 1-thio-β-D-galactoside in 2 mL of water at room temperature. The reaction was completed overnight, and the mixture was concentrated under vacuum. The residue was triturated with water and anhydrous diethyl ether and dried by treatment with petroleum ether (40−60), yielding 0.117 g (90%) of the crude product.

Synthesis of 6′-[(L-Phenylalanyl-L-phenylalanyl)amino]-hexyl 1-Thio-β-D-galactoside. 6′-[[[(9-Fluorenylmethoxy)-carbonyl]-L-phenylalanyl-L-phenylalanyl]amino]hexyl 1-thio-β-D-galactoside (0.117 g; 0.144 mmol) was dissolved at room temperature in a mixture of 2 mL of dichloromethane and 2 mL of piperidine. After 1 h, the solution was evaporated under vacuum and the residue was washed with anhydrous diethyl ether. Thereafter, the residue was dissolved in dichloromethane and precipitated with anhydrous diethyl ether, yielding 70 mg (82%) of the crude product, which was shown to contain one main impurity by TLC and RP-HPLC.

A 50 mg amount of the crude product was purified by RP-HPLC on a preparative ODS Hypersil RP18 column (particle size 5 μm, 300 × 7.8 mm) (Bischoff) using 30% acetonitrile in 0.1% TFA at a flow rate of 3 mL/min. The product was converted to its hydrochloride with hydrochloric acid and lyophilized from *tert*-butyl alcohol/water (4:1 v/v), yielding 45 mg (90%) of a voluminous crystalline white powder. The product was homogeneous by TLC and 98% pure according to analytical RP-HPLC. ES-MS, *m/z* 590.5 $[M + H]^+$.

Preparation of Cytoplasmic Membrane Vesicles. The LP overproducing strain *E. coli* T206 (Teather et al., 1980) was grown as described (Wright et al., 1983), and cytoplasmic membrane vesicles (CPM) vesicles were prepared by a variation of the procedure of Osborn et al. (1972), as described by Wright et al. (1983). The CPM vesicles were then pre-extracted with 5′-sulfosalicylate and sodium cholate (Dornmair et al., 1985; Vogel et al., 1985). Due to the remaining cholate, these vesicles were rather leaky (Dornmair et al., 1985).

Reconstitution of Lactose Permease and Preparation of Vesicles for Transport Measurements. E. coli phospholipid was extracted and purified as described (Wright et al., 1983). Lactose permease was purified from CPM vesicles of the overproducer strain *E. coli* T206 and reconstituted into vesicles of *E. coli* phospholipid to a molar lipid/LP ratio of 10 000 as described (Wright & Overath, 1984; Vogel et al., 1985). Vesicles with reconstituted LP were resuspended in 50 mM potassium phosphate, pH 7.3, and 100 mM K_2SO_4 at a lipid concentration of 10 mg/mL and subjected to two freeze−thaw cycles. Peptide galactoside (2 mM), gramicidin D (1/1000), and valinomycin (1/2000) were added, and the vesicles were extruded 31 times through polycarbonate filters (Nuclepore) of 200 nm pore size using a hand-driven liposome extruder (Avestin). This extrusion procedure (Hope et al., 1985) led to vesicles with a mean radius of 100 nm and a small size distribution, as determined by photon correlation spectroscopy using a nanosizer (Coulter N4/SD).

LP concentrations for CPM vesicles and vesicles with reconstituted LP were determined by flow dialysis with [6-^{3}H]NpαGal (Teather et al., 1980; Wright et al., 1983). Total protein was assayed using the BCA protein assay (Pierce) with bovine serum albumin as standard.

Binding Experiments. Binding studies were performed with CPM vesicles of *E. coli* strain T206 suspended in 100 mM Tris-HCl, pH 7.5, and 1 mM DTT. The dissociation constant K_d of the unlabeled peptide galactoside was determined by flow dialysis in a competition assay with tritium-labeled NpαGal (Wright et al., 1981). For this purpose, CPM vesicles (27 μM LP, 4.2 mg/mL protein) were loaded into the upper compartment of a flow dialysis chamber and preincubated with 5 or 10 μM [6-^{3}H]NpαGal (13 or 26 kBq, respectively). The lower compartment was flushed with buffer, and fractions of 1 mL/min were collected. Titration of the binding site was performed by stepwise addition of peptide galactoside. Finally, 4 mM GalSGal was added to release bound [6-^{3}H]NpαGal completely. After each titration step, five fractions were collected. Each fraction was mixed with 4 mL of scintillation liquid (Aquasafe 300 Plus, Zinsser), and the radioactivity was determined by liquid scintillation counting (Tri-Carb 300CD, Packard).

The binding of substrates to lactose permease may be asymmetric with respect to the two sides of the membrane. The competition assay for binding, however, is performed with CPM vesicles which are known to be leaky; i.e., the substrates have access to the protein from both sides of the membrane. In this case, only the average value K_d of the two dissociation constants K_d^{out} and K_d^{in} for binding from either side can be determined, with K_d given by the relation $1/K_d = 1/K_d^{out} + 1/K_d^{in}$. The binding characteristics of lactose permease with respect to two substrates, a labeled one of concentration S_l and known dissociation constant K_{dl} and an unlabeled one of concentration S_u and unknown dissociation constant K_{du}, are governed by the relation

$$T = \frac{1}{1 + S_l/K_{dl} + S_u/K_{du}} T_t \qquad (1)$$

with T denoting the concentration of free transporter and T_t the total concentration of transporter. In the binding assay, the concentration TS_l of bound labeled substrate is measured as a function of S_u for fixed S_l. The relevant expression follows from the above relation as

$$TS_l = \frac{S_l/K_{dl}}{1 + S_l/K_{dl} + S_u/K_{du}} T_t \qquad (2)$$

If the data are plotted as $(TS_l)^{-1}$ versus S_u, a straight line is obtained, the slope of which yields the dissociation constant K_{du} (Wright et al., 1981).

Transport Experiments. Transport experiments were designed to detect transport of the unlabeled peptide galactoside via counterflow of a labeled substrate. Proteoliposomes containing reconstituted LP were loaded with Phe_2-HxSGal during the extrusion procedure. In order to avoid a membrane potential, valinomycin (1/2000) and gramicidin D (1/1000) were added. In the first experiment, counterflow was started by mixing 100 μL of starting buffer (10 mM KP, 100 mM KAc, pH 5.0, 100 mM K_2SO_4, 10 μM [6,6′-$^3H_2$]GalSGal, 0.37 MBq/mL) with 1 μL of vesicle suspension (vesicles loaded with 10 mM Phe_2HxSGal). At different times, transport was stopped by the addition of 5 mL of ice-cold stopping buffer (10 mM KP, 100 mM KAc, pH 5.0, 100 mM K_2SO_4, 2 mM $HgCl_2$). In the second experiment, counterflow was started by mixing 100 μL of starting buffer (50 mM KP, pH 7.3, 100 mM K_2SO_4, 2 mM [6,6′-$^3H_2$]GalSGal, 0.37 MBq/mL) with 10 μL of vesicle suspension (loaded with 2 mM Phe_2HxSGal). Again, transport was stopped by the addition of 5 mL of ice-cold stopping buffer (50 mM KP, pH 7.3, 100 mM K_2SO_4, 2 mM $HgCl_2$). The vesicles were collected immediately on filters (type GSTF 0.22 μm, Millipore) using a vacuum filtration device (Vacuum Manifold, Amicon) and washed with 5 mL of ice-cold stopping buffer after 30 s. After another 60 s, the filters were transferred in 4 mL of scintillation liquid (Quickszint 212, Zinsser). Radioactivity was determined by liquid scintillation counting, each data point being measured in triplicate.

The time course of counterflow is complicated, and a closed expression is not available. In the second experiment, the situation simplifies, because the concentrations of the two substrates were equal and the dissociation constant of the peptide galactoside will turn out to be approximately the same as the one for GalSGal. Then counterflow may be described in analogy to exchange of one substrate in equilibrium which obeys an exponential time course (Eilam & Stein, 1974). If the experimental data are evaluated for the half-lifetime of transport, $t_{1/2}$, the transport rate for counterflow follows as

$$k_{cof} = \frac{(\ln 2)(K_d + S)V''}{t_{1/2}T_t(V' + V'')} \quad (3)$$

with V' and V'' denoting the external and internal volumes of the vesicles. The transport rate k_{cof} is determined by the individual transport rates of the two substrates, k_{cl} and k_{cu}, in a symmetric manner (Eilam & Stein, 1974):

$$\frac{1}{k_{cof}} = \frac{1}{k_{cl}} + \frac{1}{k_{cu}} \quad (4)$$

Tentatively, the experimental data will also be interpreted in terms of influx of the labeled substrate in the presence of a binding, but nontransported unlabeled substrate. The presence of the nontransported substrate is taken into account as a reduction of the effective concentration of transporters $T_{t,eff}$ participating in influx of the labeled substrate. $T_{t,eff}$ is assumed to be given by the concentration T of free transporters in the presence of the unlabeled substrate according to eq 1. Except at very small times, influx is governed by the reorientation of unloaded transporters with rate k_o, which is obtained from the half-lifetime as

$$k_o = \frac{(2 \ln 2 - 1)S_1^2 V''}{t_{1/2}K_{dl}T_{t,eff}(V' + V'')} \quad (5)$$

RESULTS

Synthesis. The peptide galactoside 6′-[(*N*-phenylalanylphenylalanyl)amino]hexyl 1-thio-β-D-galactoside (Phe_2-HxSGal) (Figure 1) was synthesized in an amount of about 100 mg and analyzed for purity.

Binding. In order to test the binding of the synthesized peptide galactoside to LP and to determine its dissociation constant, a competition assay using a labeled galactoside was employed. [6-^{3}H]NpαGal bound to LP was displaced stepwise by the unlabeled Phe_2HxSGal, and the concentration of free [6-^{3}H]NpαGal was determined by flow dialysis. The experiments were performed on LP in CPM vesicles from *E. coli* strain T206. Figure 2A shows the data of such an experiment. CPM vesicles were preincubated with [6-^{3}H]-NpαGal, and bound [6-^{3}H]NpαGal was stepwise released by the addition of Phe_2HxSGal and finally completely replaced by addition of 4 mM GalSGal (K_d = 70 μM). Above a Phe_2HxSGal concentration of 1.6 mM, no further release of [6-^{3}H]NpαGal was found. For each titration step, the amount of radioactivity in the effluent of the flow dialysis chamber was extrapolated to the start of the experiment and the concentration of bound [6-^{3}H]NpαGal was calculated.

The dissociation constant of Phe_2HxSGal was determined under the assumption that the substrate is rapidly equilibrated across the vesicle membranes due to the leakiness of the CPM vesicles. The data were linearized according to eq 2 as shown in Figure 2B. Using the value K_{dl} = 22 μM for the dissociation constant of the labeled substrate NpαGal, S_1 = 5 and 10 μM for its concentrations in the two measurements, and T_t = 27 μM for the concentration of LP, the slopes of the fitted straight lines lead to dissociation constants of Phe_2HxSGal of K_{du} = 150 μM and 160 μM in the two cases. Hence, the dissociation constant of Phe_2-HxSGal results as 150 ± 10 μM.

Transport. To test transport of Phe_2HxSGal by LP, two kinds of experiments were performed with proteoliposomes containing reconstituted LP. Both were designed as counterflow experiments in which initially Phe_2HxSGal is entrapped in the vesicles and a labeled substrate, in this case [6,6′-$^3H_2$]GalSGal, is present outside the vesicles, and the influx of the labeled substrate is detected. In the first experiment, the initial concentration of GalSGal outside the vesicles was much lower than the concentration of Phe_2-HxSGal inside the vesicles, so that an overshoot in the time course of influx should be detected, if Phe_2HxSGal is transported by LP. In the second experiment, the initial concentrations of the two substrates, GalSGal on the outside and Phe_2HxSGal on the inside of the vesicles, were equal and well above the dissociation constants of the substrates, so that this experiment should exhibit a time course similar to exchange at equilibrium, if Phe_2HxSGal is transported.

The time course of the first experiment with 10 μM GalSGal on the outside of the vesicles and 10 mM Phe_2-HxSGal inside the vesicles at pH 5 is shown in Figure 3. No transport of GalSGal was detected, indicating that LP does not catalyze transport of Phe_2HxSGal. As a control, the data for counterflow of GalSGal and lactose are included in Figure 3, exhibiting an overshoot in the internal GalSGal concentration as expected for two transportable substrates.

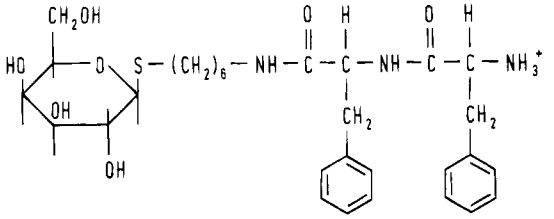

FIGURE 1: Chemical structure of the peptide galactoside 6′-[(*N*-phenylalanylphenylalanyl)amino]hexyl 1-thio-β-D-galactoside.

The time course of the second experiment with equal concentrations of 2 mM of GalSGal and $Phe_2HxSGal$ at pH 7.3 is shown in Figure 4. Influx of GalSGal is observed with a half-lifetime of $t_{1/2} = 250 \pm 100$ s. This result may be compared with GalSGal/GalSGal exchange at equilibrium under the same conditions. Such measurements have been reported in the literature and indicate that GalSGal/GalSGal exchange would be roughly 2 orders of magnitude faster (Wright, 1986; Kiefer, 1991). The conclusion is that transport of $Phe_2HxSGal$ by LP is 2 orders of magnitude slower than transport of GalSGal. The observation of a slow transport in this case is no contradiction to the first experiment, because in the first experiment the time window was a factor of 10 larger than in the second experiment and a slow transport might have escaped detection. The second experiment, however, may also be interpreted under the assumption of vanishing transport of $Phe_2HxSGal$ by LP, the observed influx of GalSGal representing a background which results from noncomplete saturation of LP by Phe_2-HxSGal on the inside of the vesicles. Unloaded LP molecules may then return to the outside of the vesicles and thus permit transport of GalSGal into the vesicles in the influx mode. To decide between these two possibilities, a quantitative analysis of the data is required.

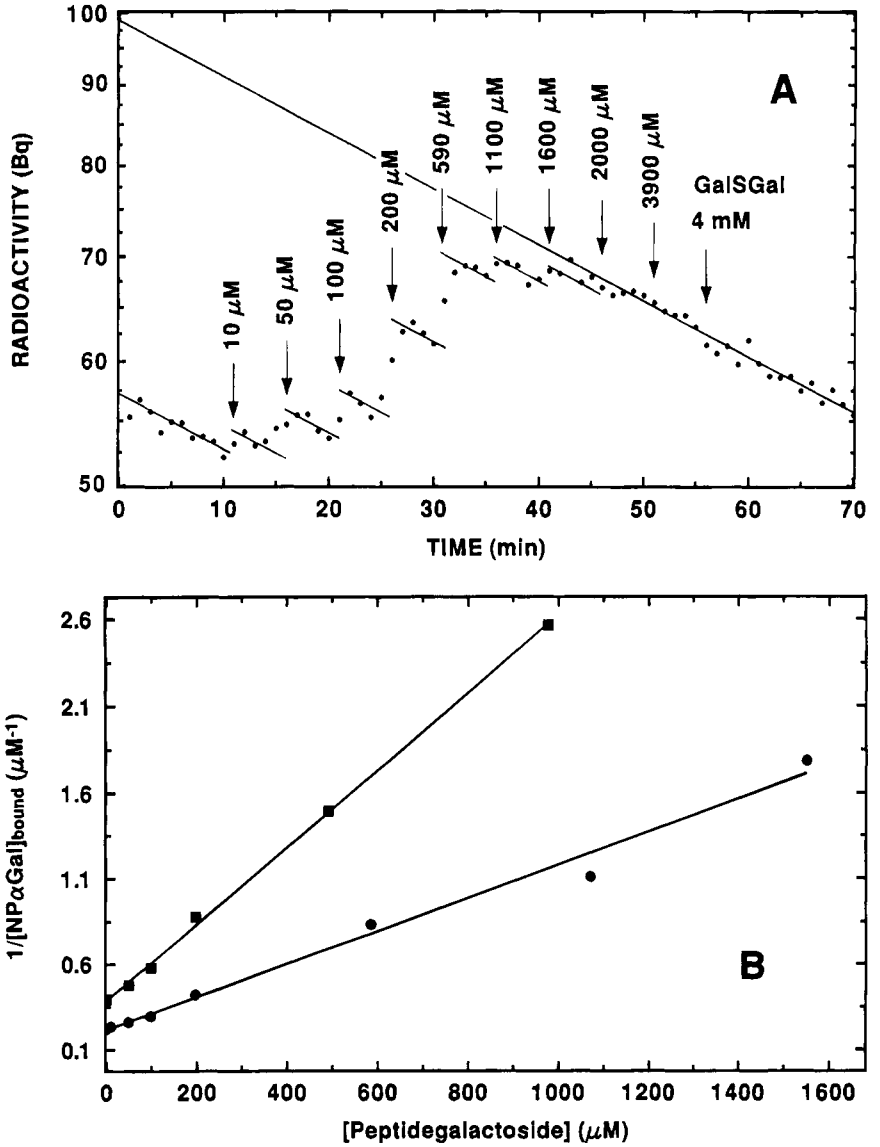

FIGURE 2: Determination of the dissociation constant of the peptide galactoside. (A) Competition of NpαGal binding by the peptide galactoside measured by flow dialysis. CPM vesicles (4.2 mg of protein/mL, 27 μM LP) were preincubated with 10 μM [6-^{3}H]NpαGal (26 kBq). NpαGal was released stepwise by the addition of peptide galactoside (concentrations as indicated) until complete replacement was reached. The subsequent points are used to construct a line corresponding to the absence of NpαGal from LP. The concentration of bound NpαGal after each titration step is reflected by the distance of the measured radioactivity from this line. (B) Linearization of the data of two flow dialysis experiments with total NpαGal concentrations of 10 μM (●) and 5 μM (■). The inverse concentration of bound NpαGal after each titration step is plotted versus the concentration of peptide galactoside according to eq 2.

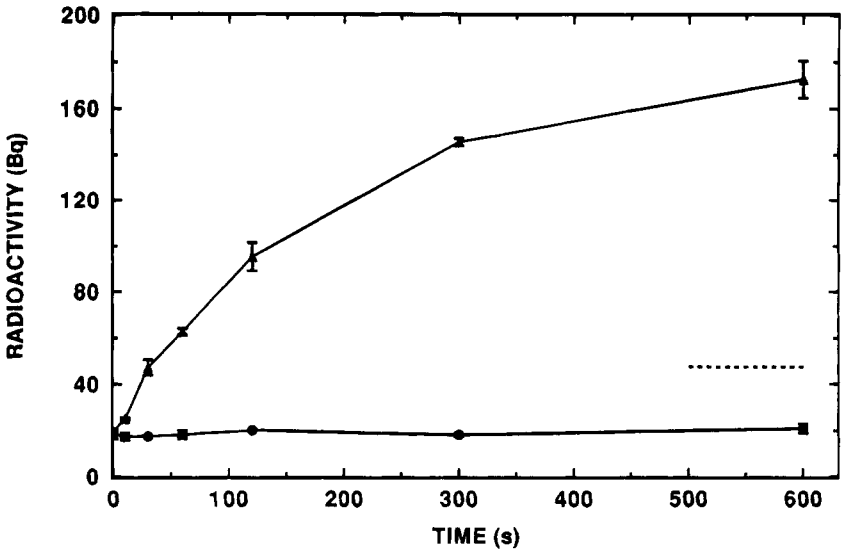

FIGURE 3: Transport of GalSGal into proteoliposomes reconstituted with LP. Proteoliposomes were prepared at pH 5.0 with 10 mM Phe_2-HxSGal inside the vesicles and 10 μM [6,6′-$^3H_2$]GalSGal outside (●). For control, Phe_2HxSGal was replaced by lactose (▲). The dashed line marks the equilibrium value of the radioactivity attained at long times in the presence of lactose but not of Phe_2HxSGal.

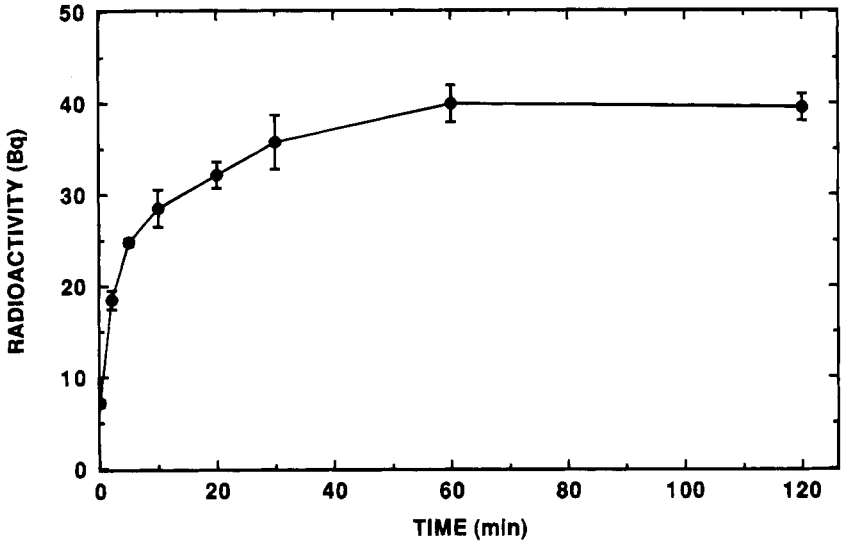

FIGURE 4: Transport of GalSGal into proteoliposomes reconstituted with LP. Proteoliposomes were prepared at pH 7.3 with 2 mM Phe_2-HxSGal inside the vesicles and 2 mM [6,6′-$^3H_2$]GalSGal outside (●).

For such an analysis, the values of three quantities will be adopted from the literature (Wright, 1986; Kiefer, 1991): the dissociation constant of GalSGal, $K_{dl} = 70$ μM, the rate for transport of GalSGal across the membrane by LP, $k_{cl} = 11$ s^{-1}, and the rate for reorientation of unloaded LP, $k_o = 3$ s^{-1}.

When the second experiment is analyzed in terms of GalSGal/Phe_2HxSGal counterflow, the dissociation constants of the two substrates GalSGal and Phe_2HxSGal, 70 μM and 150 μM, may be considered as equal in relation to their concentrations of 2 mM. Then, the second experiment may be described in analogy to exchange at equilibrium and eq 3 may be used for the analysis. Insertion of $t_{1/2} = 250$ s, $S = 2$ mM, $K_d = 100$ μM, and T_t $(V' + V'')/V'' = 270$ μM into eq 3 yields for the transport rate of counterflow $k_{cof} = 0.02$ s^{-1}. Since the rate for transport of GalSGal, $k_{cl} = 11$ s^{-1}, is much larger than k_{cof}, GalSGal/Phe_2HxSGal counterflow is rate-limited by the transport of Phe_2HxSGal and the transport rate of Phe_2HxSGal by LP follows from eq 4 as $k_{cu} = 0.02$ s^{-1}. Hence, within the framework of this interpretation, transport of Phe_2HxSGal is slower than transport of GalSGal by a factor of about 500.

When the second experiment is interpreted in terms of vanishing transport of Phe_2HxSGal and a background of GalSGal influx, one has first to estimate the fraction of unoccupied LP molecules on the inner side of the vesicles. Using eq 1 and inserting $S_l = 0$, $S_u = 2$ mM, and $K_{du} = 0.15$ mM, one obtains $T/T_t = 0.07$. This means that 7% of the LP molecules may reorient their binding site to the outside of the vesicles without substrate and thus permit influx. The effective concentration of LP is a factor of 0.07 smaller than the actual concentration. Except at very small times, influx is rate-limited by reorientation of the unloaded transporter to the outside of the vesicles and, therefore, the rate k_o is obtained from influx data. Using eq 5 and inserting $t_{1/2} = 250$ s, $S_l = 2$ mM, $K_{dl} = 70$ μM, and $T_{t,eff}$ $(V' + V'')/V'' = 0.07 \times 270$ μM $= 20$ μM, one obtains $k_o = 4.5$ s^{-1}. This value is close the literature value $k_o = 3$ s^{-1}, indicating that the second experiment may be interpreted consistently

in terms of a residual influx of GalSGal and no transport of $Phe_2HxSGal$.

Hence, the data of the second experiment are not conclusive concerning transport of the peptide galactoside. They can be interpreted under the assumption that $Phe_2HxSGal$ is not transported. Further experiments at higher concentrations of $Phe_2HxSGal$ are required for a final proof of this assumption. Certainly, the transport rate of $Phe_2HxSGal$ is at least 2 orders of magnitude smaller than that of GalSGal and 3 orders of magnitude smaller than that of lactose.

DISCUSSION

Many different galactosides with aglyconic residues ranging from the small methyl group up to the large dansyl group have so far been employed in transport studies of LP. Surprisingly, only transportable substrates of LP were found. Even for the dansyl galactosides (Schuldiner et al., 1974), transport was demonstrated (Overath et al., 1979). Our results show that a peptide galactoside with a bulky aromatic dipeptide binds to LP, but is not significantly transported. It is therefore a competitive inhibitor of galactoside transport by LP. The dissociation constant of the peptide galactoside is 150 μM, and the transport rate below 0.02 s^{-1}.

The unsubstituted aminohexyl galactoside (6′-aminohexyl 1-thio-β-D-galactoside) has very low affinity for LP. Due to protonation of the amino function, this compound is positively charged. An uncharged hexyl galactoside (6′-carboxamidohexyl β-D-thiogalactoside), on the other hand, binds to LP with an affinity comparable to that of the peptide galactoside (Schuldiner & Kaback, 1977). Finally, the dansyl galactoside DnsHxSGal has an extremely high affinity, the dissociation constant being 5 μM.

The differences for binding and transport of the galactosides may be explained in the following way: It is well-known that hydrophobic and especially aromatic groups lead to galactosides with high affinity for LP (Sandermann, 1977), and therefore, a hydrophobic pocket within the galactoside binding site has been postulated (Wright et al., 1981). In the case of DnsHxSGal, the hexyl chain and the dansyl group both contribute to the extraordinarily high affinity of the compound. By contrast, the effect of the two aromatic rings of $Phe_2HxSGal$ is much less pronounced, and the affinity is therefore lower and comparable to that of the uncharged hexyl galactoside. The aromatic dipeptide is a rather bulky structure, whereas the dansyl group is flat and probably fits better into the putative hydrophobic binding subsite. Steric effects of the bulky aromatic dipeptide may also explain why $Phe_2HxSGal$ is not transported by LP in contrast to DnsHxSGal.

At neutral pH, $Phe_2HxSGal$ is positively charged due to protonation of the α-amino group. By contrast, DnsHxSGal is a neutral compound, since the aromatic amino group is not protonated. This probably influences binding and transport. In the case of the hexyl galactoside, affinity drastically decreases with the introduction of a positive or negative charge. The affinity of the charged peptide galactoside is comparable to that of an uncharged hexyl galactoside, which may be explained in two different ways: Either the positive charge is displaced to a less sensitive region of the binding site by the introduction of the peptide, or an affinity-decreasing effect of the charge is overcompensated by a strong affinity-increasing effect of the two aromatic rings.

Finally, it is noteworthy that the peptide galactoside is the first charged substrate of LP that has been used for transport measurements. This leads to the interesting hypothesis that transport by LP is inhibited by a charged rather than a bulky aglycons. At the moment, it is not possible to decide whether the binding and transport characteristics of the peptide galactoside are determined by its size or by its charge. This question may be answered by studying different peptide galactosides. The structural variability that can be achieved with different peptide residues is a great advantage of these compounds.

ACKNOWLEDGMENT

We would like to thank G. Videnov and G. Jung from the University of Tübingen for help with the synthesis of the peptide galactoside and our colleagues P. Nollert, H. Kiefer, and T. Surrey for support with the binding and transport measurements. We furthermore appreciate the continuous interest and support of P. Overath.

REFERENCES

Anderson, G. W., Zimmerman, J. E., & Callahan, F. M. (1964) *J. Am. Chem. Soc. 86*, 1839−1842.
Appleman, J. R., & Lienhard, G. E. (1985) *J. Biol. Chem. 260*, 4575−4578.
Barrolier, J. (1961) *Naturwissenschaften 48*, 554−567.
Chipowsky, S., & Lee, Y. C. (1973) *Carbohydr. Res. 31*, 339−346.
Dornmair, K., & Jähnig, F. (1985) *Biochemistry 27*, 5008−5013.
Eilam, Y., & Stein, W. D. (1974) *Methods Membr. Biol. 2*, 283−354.
Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55−65.
Kiefer, H. (1991) Dissertation, University of Tübingen.
Osborn, M. J., Gander, J. E., Parisi, E., & Carson, J. (1972) *J. Biol. Chem. 247*, 3962−3972.
Overath, P., Teather, R. M., Simoni, R. D., Aichele G., & Wilhelm, U. (1979) *Biochemistry 18*, 1−11.
Quiocho, F. A., & Vyas, N. K. (1984) *Nature 310*, 381−386.
Sandermann, H. (1977) *Eur. J. Biochem. 80*, 507−515.
Schuldiner, S., & Kaback, H. R. (1977) *Biochim. Biophys. Acta 472*, 399−1370.
Schuldiner, S., Kerwar, G. K., Kaback, H. R., & Weil, R. (1974) *J. Biol. Chem. 250*, 1361−1370.
Teather, R. M., Bramhall, J., Riede, I., Wright, J. K., Fürst, M., Aichele, G., Wilhelm, U., & Overath, P. (1980) *Eur. J. Biochem. 108*, 223−231.
Vogel, H., Wright, J. K., & Jähnig, F. (1985) *EMBO J. 4*, 3625−3631.
Wright, J. K. (1986) *Biochim. Biophys. Acta 855*, 391−416.
Wright, J. K., & Overath, P. (1984) *Eur. J. Biochem. 138*, 497−508.
Wright, J. K., Riede, I., & Overath, P. (1981) *Biochemistry 20*, 6404−6415.
Wright, J. K., Teather, R. M., & Overath, P. (1983) *Methods Enzymol. 97*, 158−175.

BI950336Z