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Lipid Requirement of the Branched-Chain Amino Acid Transport System of *Streptococcus cremoris*†

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ABSTRACT: The role of the membrane lipid composition on the transport protein of branched-chain amino acids of the homofermentative lactic acid bacterium *Streptococcus cremoris* has been investigated. The major membrane lipid species identified in *S. cremoris* were acidic phospholipids (phosphatidylglycerol and cardiolipin), glycolipids, and glycerophosphoglycolipids. Phosphatidylethanolamine (PE) was completely absent. Protonmotive force-driven and counterflow transport of leucine was assayed in fused membranes of *S. cremoris* membrane vesicles and liposomes composed of different lipids obtained by the freeze/thaw–sonication technique. High transport activities were observed with natural *S. cremoris* and *Escherichia coli* lipids, as well as with mixtures of phosphatidylcholine (PC) with PE or phosphatidylserine. High transport activities were also observed with mixtures of PC with monogalactosyl diglyceride, digalactosyl diglyceride, or a neutral glycolipid fraction isolated from *S. cremoris*. PC or mixtures of PC with phosphatidylglycerol, phosphatidic acid, or cardiolipin showed low activities. In mixtures of PC and methylated derivatives of PE, both counterflow and protonmotive force-driven transport activities decreased with increasing degree of methylation of PE. The decreased transport activity in membranes containing PC could be restored by refusion with PE-containing liposomes. These results demonstrate that both aminophospholipids and glycolipids can be activators of the leucine transport system from *S. cremoris*. It is proposed that aminophospholipids in Gram-negative bacteria and glycolipids in Gram-positive bacteria have similar functions with respect to solute transport.

Membrane transport proteins catalyze the highly specific vectorial transfer of solutes across the membrane. Most studies on the effects of the environment on the functional properties of these proteins have focused on the water phase. Especially the effects of pH, ion composition, etc. have been studied. However, only a fraction of a membrane protein is exposed to the water phase. In order to function efficiently, a large part of the protein must be embedded in the lipid bilayer. As such, the activity of these proteins is expected to be modulated by the surrounding lipid bilayer. A number of bilayer features may be considered, such as viscosity (or lipid packing density) and charge at the lipid–water interface, parameters which are determined by the structures of individual lipids and their mutual interactions.

A limited number of reports address the question of how alterations in the lipid environment of a membrane protein affect the activities of these enzymes, and information about

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the effects of the lipid composition on bacterial transport systems is scarce (Hawrot & Kennedy, 1978; Linden et al., 1973; Overath et al., 1971; Cronan & Gelmann, 1975; Thilo et al., 1977; Ohta et al., 1977; Hirata et al., 1976; McElhaney, 1982; Chen & Wilson, 1984; Seto-Young et al., 1985; Uratani & Aiyama, 1986; Ambudkar & Maloney, 1986). The role of the polar head group of phospholipids has been extensively studied for the lactose carrier of *Escherichia coli* (Chen & Wilson, 1984; Seto-Young et al., 1985), and in less detail for the sodium-dependent leucine transport system of *Pseudomonas aeruginosa* (Uratani & Aiyama, 1986). Both transport systems appear to require phosphatidylethanolamine (PE)[1] [or phosphatidylserine (PS)] for activity. A similar requirement appears to exist for the melibiose (Wilson et al., 1985) transport system of *E. coli*. There is hardly any information available about the lipid requirement of transport systems of Gram-positive bacteria. The phosphate/sugar–phosphate exchanger of *Streptococcus lactis* ATCC 7962 (Ambudkar & Maloney, 1986) functions in the natural *S. lactis* and in *E. coli* lipids, but a clear lipid requirement could not be demonstrated.

The cytoplasmic membranes of Gram-negative and Gram-positive bacteria usually have different lipid composition. Most bacteria contain the acidic phospholipids phosphatidylglycerol (PG) and cardiolipin (CL). However, PE is often the major component of Gram-negative bacteria (Cronan & Gelmann, 1975), whereas neutral glycolipids and phosphoglycolipids are most typically constituents of Gram-positive bacteria (Fischer et al., 1978a). With respect to the distribution of PE, two groups can be discriminated among Gram-positive bacteria. One group to which *Streptococcus*, *Staphylococcus*, and *Lactobacillus* species belong (Fischer et al., 1978a) has high levels of glycolipids and no PE, while the second group which includes *Clostridium* (Johnston & Goldfine, 1983) and *Bacillus* species (Fischer et al., 1978a) contains high levels of PE and low amounts of glycolipids. In view of the reported essential role of PE in a number of transport systems of Gram-negative bacteria, it is of interest to investigate the role of the lipids in solute transport of Gram-positive bacteria in more detail.

The branched-chain amino acid carrier of the homofermentative lactic acid bacterium *Streptococcus cremoris* has been well characterized (Driessen et al., 1987a–c). This transport system catalyzes the translocation of L-leucine, L-isoleucine, and L-valine in symport with one H^+. The properties of this transport system were studied in membrane vesicles of *S. cremoris* fused with proteoliposomes containing beef heart cytochrome *c* oxidase, in order to accommodate these membranes with a functional proton pump (Driessen et al., 1985a, 1986, 1987a–d). Simultaneously with cytochrome *c* oxidase, lipids are incorporated in the streptococcal membrane. The fusion procedure has been used in this study which reports on the lipid requirement of the branched-chain amino acid carrier of *S. cremoris*. The effect of a number of natural and synthetic lipids on the counterflow and protonmotive force (Δp) driven leucine transport activity has been studied. The results indicate that the branched-chain amino acid carrier is activated by aminophospholipids and glycolipids. Since *S. cremoris* membranes are devoid of aminophospholipids, and contain high amounts of glycolipids, it is concluded that the latter are the physiological relevant lipid species required for the full function of the branched-chain amino acid carrier of *S. cremoris*. The interchangeability of glycolipids and aminophospholipids in bacterial membranes is discussed.

Experimental Procedures

Bacteria and Isolation of Membrane Vesicles. *Streptococcus cremoris* Wg2 (Prt−) was grown as described (Otto et al., 1982). Membrane vesicles were obtained by osmotic lysis (Otto et al., 1982).

Materials. Synthetic phospholipids and bovine heart and *Escherichia coli* cardiolipin were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Egg yolk phosphatidylethanolamine (PE), phosphatidyl-*N*-methylethanolamine, and phosphatidyl-*N*,*N*-dimethylethanolamine [enzymatically synthesized from egg yolk phosphatidylcholine (PC) with phospholipase D] were purchased from Avanti. Crude soybean PC (type II-S), crude *E. coli* phospholipids, egg yolk PC, wheat flour monogalactosyl diglyceride and digalactosyl diglyceride, and octyl β-D-glucopyranoside were obtained from Sigma Chemical Co. (St. Louis, MO). Octadecylrhodamine β chloride was obtained from Molecular Probes, Inc. (Junction City, OR).

Isolation of Lipids. Alternatively, *E. coli* phospholipid was extracted from *E. coli* ML 305-225 as described (Viitanen et al., 1986). *S. cremoris* lipid was extracted from cells incubated at 37 °C for 30 min in the presence of 4.2 mg/mL lysozyme (Filqueiras & Op den Kamp, 1980). In some cases, lipids were extracted from cells of *S. cremoris* grown on sodium acetate free complex medium in the presence of 2.2 μM [U-^{14}C]acetic acid (4.7 TBq/mol). Crude *E. coli*, *S. cremoris*, and soybean phospholipids were acetone/ether washed as described (Viitanen et al., 1986; Kagawa & Racker, 1971). Soybean PE was isolated as described (Kagawa et al., 1973). Crude *S. cremoris* lipids were fractionated as described (Vorbeck & Marinetti, 1965). Dried silica gel (100 g of Kieselgel 60, 7–230 mesh; Merck, Darmstadt, FRG) was resuspended in chloroform and poured in a glass column with a diameter of 4 cm. The column was washed with chloroform, and 1 g of crude *S. cremoris* lipid dissolved in 10 mL of chloroform/methanol (9:1 v/v) was loaded on the column. Neutral lipids, neutral glycolipids, and acidic phospholipids were subsequently eluted with 450 mL of chloroform, 150 mL of acetone, and 250 mL of methanol, respectively. Fractions were analyzed by two-dimensional thin-layer chromatography as described below. Lipids were dissolved in chloroform/methanol (3:1 v/v) and stored at −20 °C under N_2.

Lipid Composition Analysis. Lipids were separated by two-dimensional thin-layer chromatography on precoated silica gel plates (Merck, Kieselgel 60) using the following solvent systems: A, chloroform/methanol/ammonia/water (90:54:5.5:5 v/v); B, chloroform/methanol/acetic acid/water (90:40:12:2 v/v). Soybean phospholipid and mono- and digalactosyl diglycerides, were chromatographed in parallel as reference compounds. Chromatograms were stained with (i) I_2 vapor for lipids, (ii) α-naphthol/H_2SO_4 for carbohydrates (Jacin & Mischkin, 1965), and (iii) ninhydrin reagent to detect amino groups. Phospholipid phosphorus was determined by the method of Rouser (Rouser et al., 1970). The lipid composition of *S. cremoris* membranes was quantitated by the amount of radioactivity incorporated in the acyl chains. Carbohydrate content was determined by the anthrone pro-

[1] Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; DOPE, dioleoylphosphatidylethanolamine; DOPS, dioleoylphosphatidylserine; DOPA, dioleoylphosphatidic acid; CL, cardiolipin; GL, glycolipid; MGDG, monogalactosyl diglyceride; DGDG, digalactosyl diglyceride; *N*-methyl-PE, phosphatidyl-*N*-methylethanolamine; *N*,*N*-dimethyl-PE, phosphatidyl-*N*,*N*-dimethylethanolamine; R_{18}, octadecylrhodamine β chloride; PIPES, sodium piperazine-*N*,*N*′-bis(2-ethanesulfonate); PI, phosphatidylinositol; DPH, 1,6-diphenyl-1,3,5-hexatriene.

cedure after hydrolysis in 2 M HCl at 100 °C for 2.5 h (Fischer & Seyferth, 1968; Fischer, 1977a). D-Glucose was used as a standard.

Analysis of Fatty Acids. Fatty acids were analyzed as methyl esters (Morrison & Smith, 1964) by gas–liquid chromatography using a 3% Silar 5 CP column on 80/100 Gaschromosorb Q_{11} operated at 180 °C with N_2 as carrier gas.

Preparation of Liposomes and Fusion of Liposomes with S. cremoris Membrane Vesicles. Lipids dissolved in chloroform/methanol (3:1 v/v) were mixed together in appropriate proportions and dried under a stream of N_2 gas. Traces of solvent were removed under vacuum for 1 h. The dried lipid was suspended in 50 mM potassium phosphate (pH 6.0) at a concentration of 20 mg of lipid/mL and dispersed by ultrasonic irradiation using a bath sonicator (Sonicor, Sonicor Instruments, New York, NY). Liposomes were obtained by sonication (probe type; MSE Scientific Instruments, West Sussex, U.K.) for 300 s at maximal amplitude, using intervals of 15-s sonication and 45-s rest, at 4 °C under a constant stream of N_2. Fusion of liposomes with *S. cremoris* membrane vesicles was performed by freeze/thaw–sonication as described previously (Driessen et al., 1985a, 1986). *S. cremoris* membrane vesicles (2 mg of protein) and liposomes (20 mg of lipid) were mixed in a final volume of 1 mL of 50 mM potassium phosphate (pH 6.0). The suspension was rapidly frozen into liquid N_2 and stored until use. Frozen membranes were slowly thawed and sonicated for 8 s at 4 °C with a probe sonicator.

Fusion Assay. Fusion was quantitated with the R_{18} (octadecylrhodamine β chloride) fusion assay (Hoekstra et al., 1984). *S. cremoris* membrane vesicles were labeled with R_{18} (4 mol % phospholipid phosphorus) as described (Driessen et al., 1986). Labeled membranes were fused with liposomes of different lipid composition as described above. R_{18} fluorescence was determined prior and after the addition of 1% (v/v) Triton X-100 (Hoekstra et al., 1984). Excitation and emission were performed at 560 and 590 nm, respectively. The maximal level of fluorophore dilution was determined as follows: fused membranes were solubilized by the addition of octyl β-D-glucopyranoside (30 mM final concentration), and subsequent reconstitution was accomplished by detergent dialysis (Driessen et al., 1985b). The intensity at maximal fluorophore dilution was set at 100%, and the intensity of the labeled membrane vesicles was taken as the zero level.

Transport Assays. L-Leucine counterflow was performed as follows: fused membranes were incubated for 1 h at 25 °C with 50 mM potassium phosphate (pH 6.0) supplemented with 1 mM L-leucine. Loaded membranes were collected by centrifugation for 1 h at 55 000 rpm (maximally 280000*g*) in a Beckman type 75 Ti rotor at 5 °C. Aliquots of 4 μL (approximately 8 mg of protein/mL) were diluted into 200 μL of 50 mM potassium phosphate (pH 6.0) containing 1.5 μM L-[U-^{14}C]leucine (final concentration approximately 20 μM). At intervals, 2 mL of ice-cold 0.1 M LiCl was added, and the sample was filtered immediately on a 0.45-μm cellulose nitrate filter (Millipore). Radioactivity was determined by liquid scintillation spectrophotometry. Δp-driven L-leucine transport was performed essentially as described (Driessen et al., 1986). Fused membranes were incubated for 1 h at 25 °C with 20 mM potassium phosphate (pH 6.0) supplemented with 100 mM potassium acetate, in the presence of 2 nmol of valinomycin/mg of protein. Aliquots (2 μL) of a concentration membrane suspension (approximately 8 mg of protein/mL) were subsequently diluted into 200 μL of 20 mM sodium phosphate (pH 6.0) supplemented with 100 mM sodium piperazine-*N*,*N*′-bis(2-ethanesulfonate) (PIPES) and 1.5 μM L-[U-^{14}C]leucine. Uptake was assayed as described above. For kinetic analysis of leucine uptake, rates were determined from the uptake values obtained after 5-s incubation. A concentration range between 0.75 and 20 μM leucine was used. Results were analyzed by Eady–Hofstee plots. Transport was assayed at 25 °C.

Table I: Lipid Composition of *S. cremoris* Membranes

phospholipid	%[a]	glycolipid	%[a]
phosphatidylglycerol	14.1	glycerophosphoglycolipid	21.8
cardiolipin	31.7	dihexosyl diglyceride	22.6
lysophosphatidylglycerol	3.2	monohexosyl diglyceride	3.0
unidentified	3.3	unidentified	0.3
	total 52.3		total 47.7

[a] Lipid composition was determined from the amount of [^{14}C]acetate incorporated. Lipids were identified as described under Experimental Procedures.

Other Analytical Procedures. The electrical potential across the membrane ($\Delta\psi$, interior negative) was determined from the distribution across the membrane of the lipophilic cation tetraphenylphosphonium by using a tetraphenylphosphonium-selective electrode as described (de Vrij et al., 1986). Trapped volume measurements were performed with the fluorophore calcein as described (Oko et al., 1982). Membranes were fused in the presence of 100 μM calcein, as described above. Calcein fluorescence was determined prior and after the addition of 100 μM $CoCl_2$ in order to quench external calcein. Excitation and emission were performed at 480 and 520 nm, respectively. Protein was determined by the method of Lowry et al. (1951) in the presence of sodium dodecyl sulfate (Dulley & Grieve, 1975). Bovine serum albumin was used as standard.

Results

Lipid Composition of S. cremoris Membranes. The lipid composition of the membrane of *S. cremoris* Wg2 was examined. Cells were grown in the presence of [^{14}C]acetic acid and treated with lysozyme, and the lipids were extracted with chloroform/methanol. Crude lipid extracts were analyzed by two-dimensional thin-layer chromatography. As shown in Table I, *S. cremoris* membranes contain the phospholipids cardiolipin and phosphatidylglycerol and high amounts of neutral glycolipids. In addition, phosphorus-containing glycolipids were detected, tentatively identified as glycerophosphoglycolipids (Fischer et al., 1978a,b).

Fusion Efficiency and Trapped Volume. Liposomes were prepared with different lipid composition and fused with *S. cremoris* membrane vesicles by the freeze/thaw–sonication technique. The efficiency of fusion between *S. cremoris* membrane vesicles and liposomes of different phospholipid composition was quantitated with the R_{18} fusion assay. A high extent of fusion was observed with all different phospholipid vesicles, except for DOPC and soybean lipid which showed a lower fusion efficiency (Table II). In order to test if closed membrane structures were obtained after membrane fusion, the internal volume of the fused membranes with different phospholipid composition was determined. The internal volume was estimated from the trapped amount of the fluorophore calcein (Table II) and was equivalent to 6.5–7.5 μL of water space/mg of protein for each of the different lipid mixtures. A somewhat lower value was found with DOPC/DOPA (1:3 v/v). These results show that there are no dramatic differences in fusion and trapping efficiency between the different lipid mixtures, indicating that with all different lipid mixtures closed membrane structures are obtained upon fusion.

Table II: Trapping Volume and Fusion Efficiency[a]

composition of liposomes	fusion efficiency (%)	calcein trapping[b] volume (μL/mg of protein)
soybean lipid	74.7 ± 6.2	7.9 ± 0.4
E. coli lipid	84.2 ± 5.1	7.3 ± 0.5
S. cremoris lipid	79.2 ± 4.7	7.3 ± 0.4
DOPC	73.2 ± 5.7	7.9 ± 0.4
DOPC/DOPE (1:3 w/w)	93.1	6.9
DOPC/DOPS (1:3 w/w)	94.7	7.1
DOPC/DOPG (1:3 w/w)	94.2	6.7
DOPC/DOPA (1:3 w/w)	89.7	5.9
DOPC/*E. coli* CL (1:3 w/w)	97.8	7.3
DOPC/*S. cremoris* GLC (3:2 w/w)	87.5	6.9

[a] Fusion efficiency was determined by using the R_{18} fusion assay. [b] Trapping volume of fused membranes was determined as described under Experimental Procedures. The mean value of at least two determinations is indicated.

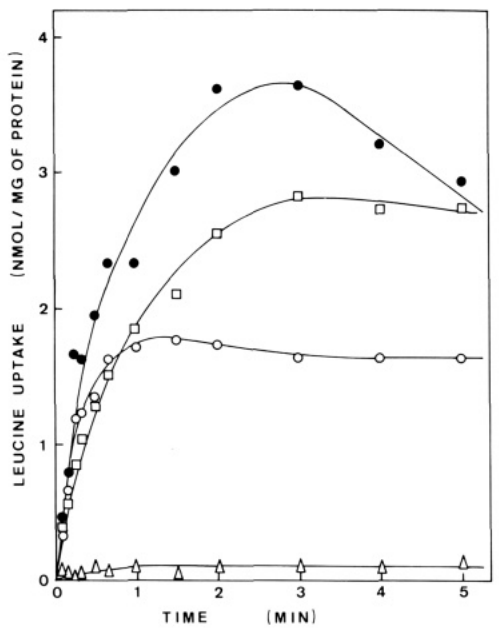

FIGURE 1: Counterflow of leucine in fused membranes composed of soybean (○), *S. cremoris* (□), and *E. coli* (●) lipid. Uptake of leucine by fused membranes composed of soybean lipid (△) not loaded with leucine.

Leucine Transport in Fused Membranes Composed of Natural Lipid Mixtures. Liposomes were prepared from soybean, *E. coli*, and *S. cremoris* phospholipids and fused with *S. cremoris* membrane vesicles. Leucine transport activity was assayed by the counterflow technique. For this purpose, fused membranes were loaded with 1 mM leucine and then diluted 50-fold into a solution containing 1.5 μM [^{14}C]leucine. Under those conditions, an outwardly directed leucine concentration gradient was imposed which resulted in accumulation of [^{14}C]leucine due to rapid label exchange. Accumulation of label was only transient since release of intravesicular leucine via the carrier led to a gradual drop in its internal concentration and finally to equilibration. High rates of leucine transport were observed with all three lipid mixtures (Figure 1), although *E. coli* phospholipid was found to be most effective in stimulating transport. Only a low level of leucine uptake was observed with fused membranes which were not preloaded with leucine (Figure 1). The kinetic constants of leucine transport were determined in the presence of an artificially imposed Δp. Fused membranes were incubated in a suspension containing 20 mM potassium phosphate (pH 6.0), 100 mM potassium acetate, and the K^+ ionophore valinomycin. Subsequently, a Δp was imposed by diluting the membranes into a solution containing 20 mM sodium phosphate (pH 6.0) and 100 mM sodium PIPES. In this way, both a pH gradient and also a membrane potential were established. High maximal rates of leucine uptake were observed with all three natural lipid mixtures, with similar K_t values (Table III).

Table III: Kinetic Constants of Δp-Driven L-Leucine Transport by Membrane Vesicles of *S. cremoris* Fused with Liposomes Prepared from Natural Lipid Mixtures[a]

lipid source	K_t (μM)	V_{max} [nmol min^{-1} (mg of protein)$^{-1}$]
soybean	4.1	16.7
S. cremoris	3.7	25.5
E. coli	4.4	34.3

[a] Kinetic constants were determined as described under Experimental Procedures.

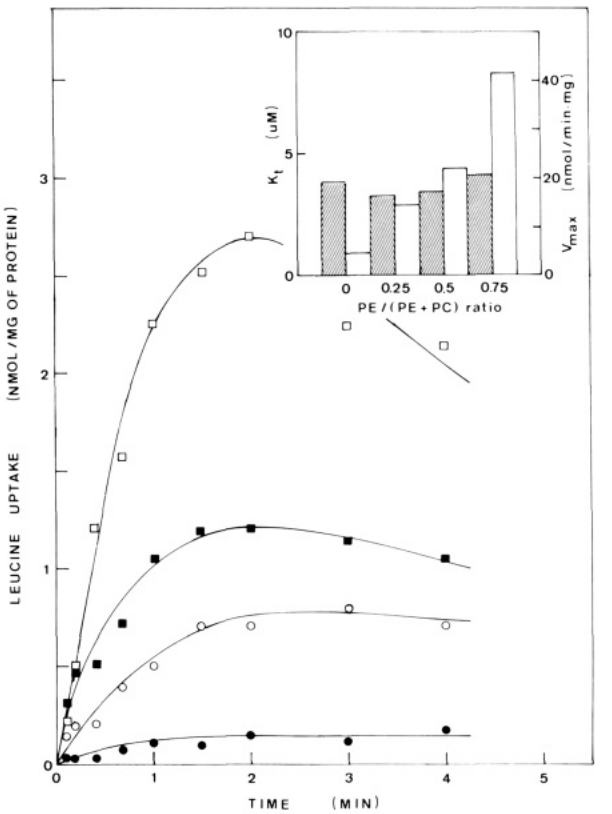

FIGURE 2: Leucine counterflow in fused membranes composed of different mixtures of soybean phosphatidylethanolamine (PE) and egg yolk phosphatidylcholine. Fused membranes with PC alone (●), PC/PE (3:1 w/w) (○), PC/PE (1:1 w/w) (■), and PC/PE (1:3 w/w) (□). Inset: Effect of the PE:(PE + PC) ratio (w/w) on the kinetic parameters of Δp-driven leucine transport. K_t (filled column) and V_{max} (white column).

E. coli phospholipids contain approximately 65% phosphatidylethanolamine (PE), 30–35% phosphatidylglycerol (PG), and 0–5% cardiolipin (CL) [which is in accordance with Otha et al. (1981)]. On the other hand, soybean phospholipids contain approximately 25% PE, 38% phosphatidylcholine (PC), 17% phosphatidylinositol (PI), 11% phosphatidic acid (PA), and trace amounts of CL and PG [which is in accordance with Kagawa et al. (1973)]. The common component appears to be PE. Leucine transport was therefore assayed in fused membranes containing isolated soybean PE. Since unsaturated PE's like soybean PE do not readily form bilayer structures, mixtures of soybean PE and egg yolk PC were used. Leucine counterflow activity increased with increasing PE content of the fused membranes (Figure 2). Similar results were obtained for Δp-driven leucin transport (inset of Figure 2). Low

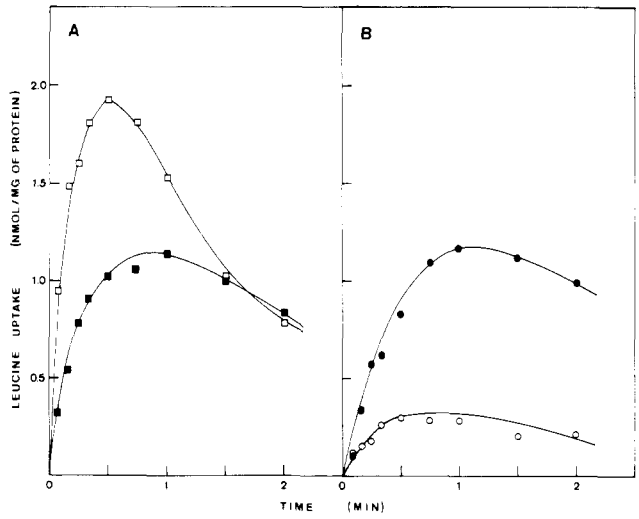

FIGURE 3: Reversible activation and inactivation of Δp-driven leucine transport by fused membranes composed of mixtures of soybean PE and egg yolk PC. (A) Membrane vesicles of *S. cremoris* (1 mg of protein) fused with PE/PC (1:3 w/w) liposomes (10 mg of lipid) (□) and refused with PC liposomes (■) (10 mg of lipid). (B) Membrane vesicles of *S. cremoris* fused with PC liposmes (○) and refused with PE/PC (1:3 w/w) liposomes (●).

activity was observed with PC alone. The stimulatory influence of PE is due to an effect on the maximal rate of uptake (V_{max}) and not on K_t (inset of figure 2). Similar results were obtained with isolated *E. coli* PE (data not shown).

The reversibility of inactivation/activation of the leucine transport system by egg yolk PC and soybean PE was investigated. Membranes of *S. cremoris* were fused with PC liposomes. The PE content of these inactive fused membranes was enhanced by refusion with liposomes composed of PE/PC (3:1 w/w), resulting in a final PE:PC ratio of 0.6. By this treatment, reactivation of Δp-driven leucine transport was observed (Figure 3B). In another experiment, membranes were first fused with PE/PC (3:1 w/w) liposomes. Subsequently, the PE content was reduced by refusion with PC liposomes, giving a final PE:PC ratio of 0.6. This treatment resulted in a depression of Δp-driven leucine transport (Figure 3A).

Transport in Fused Membranes Prepared from Different Synthetic Phospholipids. In the experiments with mixtures of soybean PE and egg yolk PC, a decrease in membrane fluidity, as measured with 1,6-diphenyl-1,3,5-hexatriene (DPH), was observed with increasing soybean PE content of the fused membranes (T. Zheng, unpublished results). In order to minimize effects of membrane fluidity and/or fatty acid acyl chain, liposomes were prepared from mixtures of synthetic phospholipids with a monounsaturated fatty acid chain (9-*cis*-octadecanoic acid). The phase transition temperature of these phospholipids is far below the transport assay temperature of 25 °C. Liposomes were prepared from DOPC, DOPC/DOPE (1:3 w/w), DOPC/DOPG (1:3 w/w), DOPC/DOPA (1:3 w/w), and DOPC/DOPS (1:3 w/w) and fused with *S. cremoris* membrane vesicles. In all cases, closed membrane vesicles are obtained upon fusion (Table II). High rates of leucine transport, both Δp-driven (Figure 4A) and also counterflow (Figure 4B), were observed with fused membranes containing DOPE and DOPS. Low activities were observed with DOPC alone, or with mixtures of DOPC with DOPG or DOPA

One of the possible explanations of the inability of certain lipids to support leucine transport could be a deficiency in the ability to maintain an artificially imposed Δp. The membrane

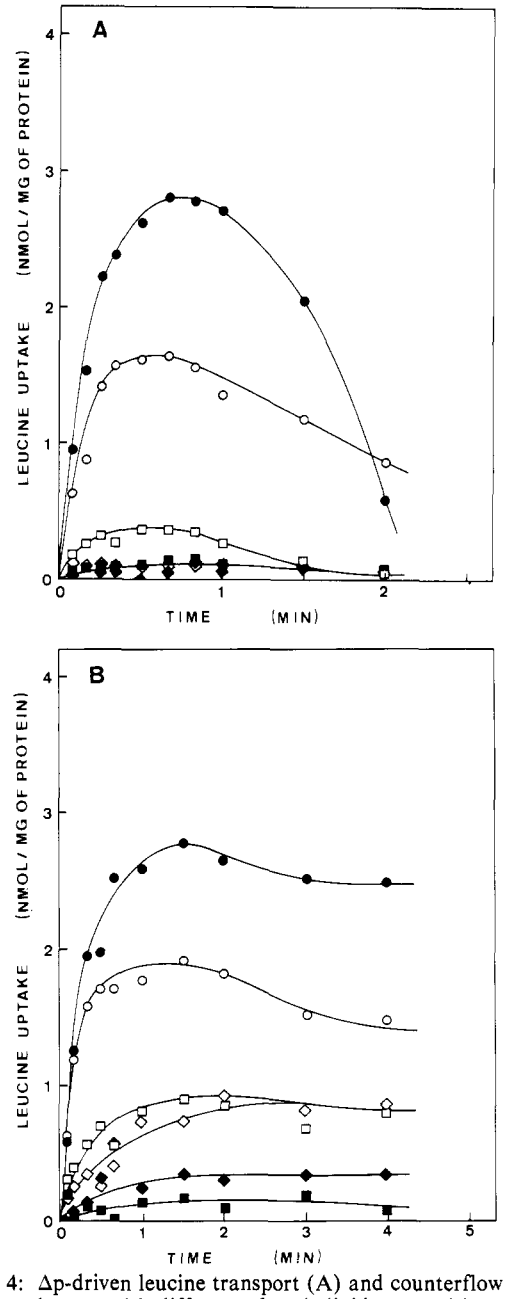

FIGURE 4: Δp-driven leucine transport (A) and counterflow (B) in fused membranes with different phospholipid composition. Fused membranes with DOPC alone (♦), DOPC/DOPS (1:3 w/w) (●), DOPC/DOPE (1:3 w/w) (○), DOPC/DOPG (1:3 w/w) (□), DOPC/DOPA (1:3 w/w) (■), and DOPC/*E. coli* CL (1:3 w/w) (◇).

potential generated by the potassium diffusion gradient in the fused membranes of different phospholipid composition was estimated from the distribution of the lipophilic cation tetraphenylphosphonium. The calculated $\Delta\psi$ was very similar for the different preparations (e.g., −100 to −115 mV) (data not shown).

Role of Cardiolipin. *S. cremoris* membranes contain large amounts of cardiolipin (CL) whereas PE and PS are completely absent (Table I). The effect of CL on leucine transport was studied. Since synthetic CL is not commercially available, CL obtained from a natural source had to be used. The acyl chain composition of *S. cremoris* CL is approximately 7% C14:0, 6% C14:1, 15% C16:0, 6% C16:1, 30% C18:1, 6% C18:3, and 16% $C19_c$, giving a mean number of unsaturated groups per acyl chain ($\sum_{unsat}/\sum_{total}$) of about 0.59 (data not shown). *E. coli* CL, obtained from Avanti, contains 29% cyclopropane-containing acyl chains, in addition to 32% C16:0,

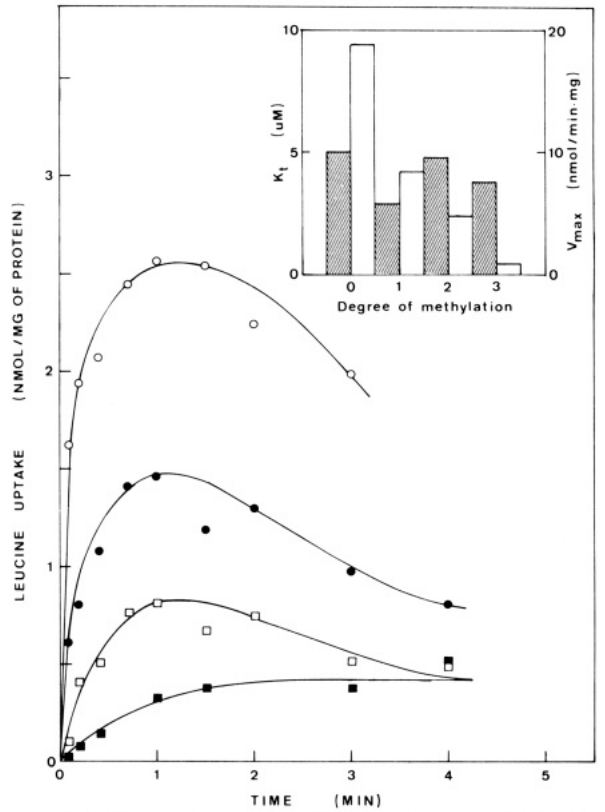

FIGURE 5: Effect of degree of methylation of egg yolk phosphatidylethanolamine on leucine counterflow. Fused membranes with PC/PE (1:3 w/w) (○), PC/*N*-methyl-PE (1:3 w/w) (●), PC/*N*,*N*-dimethyl-PE (1:1 w/w) (□), and PC alone (■). Inset: Effect of the degree of methylation of PE on the kinetic parameters of Δp-driven leucine transport. K_t (filled column) and V_{max} (white column).

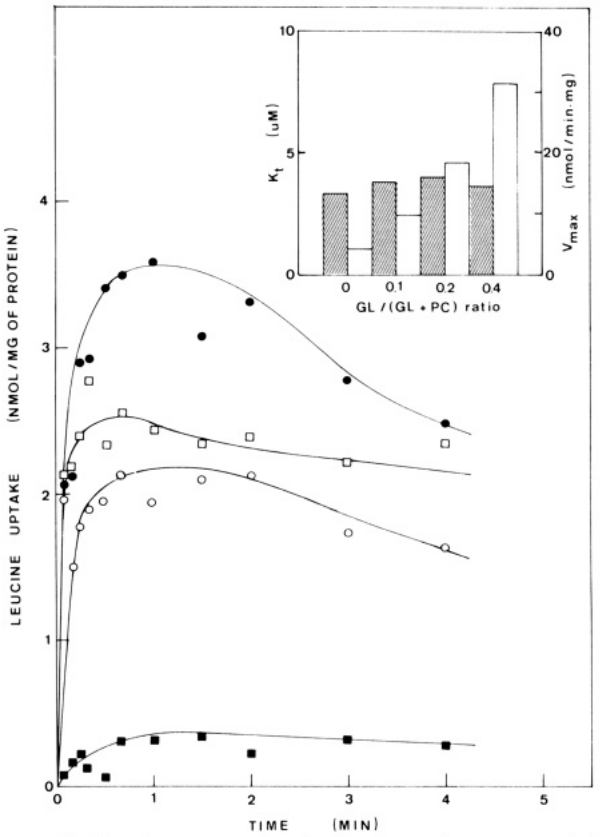

FIGURE 6: Leucine counterflow in fused membranes containing glycolipids. Fused membranes with DOPC/MGDG (3:2 w/w) (●), DOPC/DGDG (3:2 w/w) (○), DOPC/*S. cremoris* glycolipid (3:2 w/w) (□), and DOPC alone (■). Inset: Effect of the GL:(GL + DOPC) ratio (w/w) on the kinetic parameters of Δp-driven leucine transport. K_t (filled column) and V_{max} (white column).

1% C18:0, and 38% C18:1 ($\sum_{unsat}/\sum_{total}$ of approximately 0.38) (Avanti Polar Lipids, product information). On the other hand, bovine heart CL (Avanti) contains more unsaturated acyl groups (87% C18:2, 6% C18:3, and trace amounts of C16:0, C16:1, C18:0, and C18:1; $\sum_{unsat}/\sum_{total}$ = 1.96) but no cyclopropane-containing acyl chains. Therefore, leucine transport activity was studied with fused membranes containing mixtures of *E. coli* CL and DOPC. Low transport activities (both Δp as well as counterflow) were observed with CL-containing membranes (Figure 4A,B). Similar results were obtained with bovine heart CL or mixtures of bovine heart CL and DOPG (data not shown). Δp-driven leucine transport in mixtures of bovine heart CL and soybean PE decreased with increasing amounts of CL in the fused membranes (data not shown).

Effect of Methylation of PE on Leucine Transport. In order to analyze the effect of the polar head group on leucine transport in more detail, fused membranes were prepared with phospholipids of increasing degree of N-methylation from PE to PC. PE, *N*-methyl-PE, and *N*,*N*-dimethyl-PE were enzymatically synthesized from egg yolk PC with phospholipase D. Liposomes were prepared from PC, *N*,*N*-dimethyl-PE/PC (3:1 w/w), *N*-methyl-PE/PC (3:1 w/w), and PE/PC (3:1 w/w). The results indicate that both Δp-driven transport (inset of Figure 5) and counterflow (Figure 5) activities increase with decreasing degree of methylation. The V_{max} of Δp-driven leucine transport decreased with increasing degree of methylation, while K_t remains essentially unaffected (inset of Figure 5).

Leucine Transport in Fused Membranes Containing Glycolipids. Leucine transport is inactive in CL or CL/DOPG liposomes. Therefore, the effect of glycolipids, present in large quantities in *S. cremoris* membranes (Table I), was studied. Leucine transport was assayed with fused membranes containing the wheat flour glycolipids monogalactosyl diglyceride (MGDG) or digalactosyl diglyceride (DGDG). MGDG does not readily form bilayer structures. Closed membrane vesicles were obtained when *S. cremoris* membranes were fused with liposomes prepared from mixtures of DOPC/MGDG (3:2 w/w) and DOPC/DGDG (3:2 w/w) (Table II). As demonstrated in Figure 6, high rates of leucine counterflow were observed with these lipid mixtures. Similar results were obtained when instead of DOPC mixtures of glycolipids with CL were used (data not shown). The neutral glycolipids (GL's) from *S. cremoris* were isolated by silica gel column chromatography. Crude *S. cremoris* lipid was separated into three distinct fractions: (i) fatty acids eluted with chloroform; (ii) GL eluted with acetone; and finally (iii) a fraction eluted with methanol which contained CL, PG, and the glycerophosphoglycolipids (data not shown). One gram of crude *S. cremoris* lipid typically yielded about 135 mg of GL and 490 mg of phosphorus-containing lipids, which agrees reasonably well with the lipid composition shown in Table I. The GL fraction was slightly contaminated with glycerophosphoglycolipids (less than 5% on sugar content). *S. cremoris* membranes were fused with liposomes prepared from a mixture of DOPC/*S. cremoris* GL (3:2 w/w), and the leucine counterflow activity was assayed. Rates of leucine transport with membranes containing *S. cremoris* GL were similar to those observed with MGDG or DGDG (Figure 6). The V_{max} of Δp-driven leucine uptake increased with increasing GL content of the fused membranes, while K_t remained unaffected (inset of Figure 6). The fusion efficiency and the internal volume of glycolipid-containing

fused membranes were very similar to those observed with other lipids (Table III). These results demonstrate that glycolipids are the physiological relevant lipid species for activating the leucine carrier of *S. cremoris*.

Discussion

In this study which reports on the lipid requirement of the branched-chain amino acid carrier of *S. cremoris*, the lipid composition was manipulated by membrane fusion. In most studies on the lipid requirement of transport systems, the carrier is extracted from the membrane by detergents in the presence of different phospholipids, followed by reinsertion of the transport system into liposomes of different composition. A limitation of this approach is that differences observed between the various lipids might be due to variability in the incorporation efficiency of the transport protein into proteoliposomes of different lipid composition. For instance, the presence of CL during the isolation of a number of mitochondrial transport systems greatly enhances the extraction of the carrier, but CL seems not always to be necessary for the preparation of active proteoliposomes (Wohlrab, 1987). By the use of the 4-nitro[2-^{3}H]phenyl α-D-galactopyranoside labeled lactose carrier, it could be demonstrated that there was no difference in the incorporation efficiency of the lactose carrier with different lipids (Chen & Wilson, 1984; Seto-Young et al., 1985). However, for most transport systems, such an affinity label is not available. On the other hand, membrane fusion provides a relatively simple method to modify the lipid composition without the need to extract the protein from the lipid bilayer. In this way, the number of transport systems can be kept constant, which allows a direct estimation of carrier activity in different lipid bilayers. Furthermore, the fusion efficiency is rather independent of the lipid composition of the liposomes (Pick, 1981) (Table II). Another advantage of this technique is that no detergents have to be used. A disadvantage of this method is that some endogenous lipid remains present in the fused membranes.

Two types of carrier activity have been examined; counterflow of leucine and Δp-driven leucine transport. The initial phase of counterflow represents the exchange activity of the carrier. Since this activity is not related to the magnitude of Δp, it provides a direct indication of the carrier activity. Among the crude lipid extracts, *E. coli* lipid was most effective. A systematic investigation on how the lipid composition of a bilayer affects the catalytic activity of a membrane protein is only possible when a single feature of the bilayer is altered. Therefore, experiments have been performed with synthetic phospholipids with fixed lipid acyl chain composition. High carrier activity was only observed with PE and PS (Figure 4), whereas PC, PA, and PG were inactive, despite high fusion efficiencies (Table III). These dramatic variations exclude the possibility that trace amounts of natural *S. cremoris* lipid (approximately 5–8% total lipid) are sufficient for fully activating the leucine carrier. In all cases, tightly closed fused membranes were obtained as indicated by the trapping volume of calcein (Table III) and the ability to maintain an artificially imposed Δp (data not shown). It should be emphasized that Δp-driven leucine uptake showed qualitatively a similar lipid dependence as counterflow. The kinetic experiments demonstrate that the different lipids affect V_{max}, whereas K_t remains largely unaffected. PE is the active lipid species of natural *E. coli* and soybean lipid. However, PE is completely absent in membranes of *S. cremoris* (Table I) (Fischer et al., 1978a). This appears to be at variance with the observation that natural *S. cremoris* lipids are almost as effective as *E. coli* lipids in stimulating leucine transport. The major phospholipid of *S. cremoris* membranes, CL failed to activate leucine transport. The reason for this cannot be found in the acyl chain composition of CL. Both the highly unsaturated bovine heart CL and *E. coli* CL were inactive.

About 50% of the *S. cremoris* membrane lipids are glycolipids and glycerophosphoglycolipids (Table I) (Fischer et al., 1978a). The leucine carrier is highly active in fused membranes containing GL. The sarcoplasmic reticulum Ca^{2+}-ATPase (Navarro et al., 1984) is dramatically influenced by the degree of glycosylation of GL. Such an effect is not observed for the leucine carrier of *S. cremoris*. Both monogalactosyl diglyceride and digalactosyl diglyceride were found to be active. Streptococci also contain a large amount of the unusual glycerophosphoglycolipids (Table I). These derivatives of phosphatidylglycerol have been well characterized by Fischer and co-workers (Fischer et al., 1978a,b; Fischer, 1977a,b). It was suggested that they function as the lipid anchor of cell wall lipoteichoic acid (Ganfield & Pieringer, 1975). No direct evidence is available with respect to the role of this glycolipid in leucine transport. It should be stressed that the phospholipid fraction obtained by silica gel chromatography was devoid of neutral glycolipids and enriched in glycerophosphoglycolipids. This fraction was also highly active in stimulating leucine transport, but the activity could be increased by adding neutral glycolipids (unpublished).

MGDG and unsaturated PE's, such as DOPE and soybean PE, are able to form nonbilayer structures (de Kruijf et al., 1985). Our results do not support the idea that membrane proteins display an absolute requirement for cone-shaped lipids. Unlike the activity of the Ca^{2+}-ATPase of sarcoplasmic reticulum (Navarro et al., 1984), the activity of the leucine transport protein was also observed with the bilayer lipids PS and DGDG. This activity decreased with increasing degree of methylation from PE to PC. Both PE and PS activate leucine transport, indicating that this transport activity depends on the amino group in the phospholipid, similar to the lactose carrier of *E. coli* (Chen & Wilson, 1984). The fact that also glycolipids activate the carrier as well suggests that these lipids have similar properties. On the basis of the distribution of glycolipids and PE among Gram-positive and Gram-negative bacteria, it has been suggested that these lipids have similar functions in lipid bilayers (Minnikin et al., 1971). Both lipid species are able to form hydrogen bonds. Hydrogen bonding might play an important role in the lipid–protein interaction of the branched-chain amino acid carrier. However, the hydroxyl group in the head-group region of PG is also expected to be able to form hydrogen bonds. PG did not activate the leucine or lactose carrier (Chen & Wilson, 1984). The position of the hydroxyl group within the molecule might render it inactive for lipid–protein interaction. In contrast, leucine transport in *Pseudomonas aeruginosa* (Uratani & Aiyama, 1986) was affected by PE and PG.

The findings demonstrate that the amino groups in PE and PS are important for activation of the branched-chain amino acid carrier. A similar requirement is fulfilled by glycolipids, which are the physiological relevant lipid species. It is suggested that aminophospholipids and glycolipids play an interchangeable role in the activation of solute transport proteins.

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