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Hydrophobic membrane thickness and lipid–protein interactions of the leucine transport system of *Lactococcus lactis*

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The effect of the phospholipid acyl chain carbon number on the activity of the branched-chain amino acid transport system of *Lactococcus lactis* has been investigated. Major fatty acids identified in a total lipid extract of *L. lactis* membranes are palmitic acid (16:0), oleic acid (18:1) and the cyclopropane-ring containing lactobacillic acid (19ν). *L. lactis* membrane vesicles were fused with liposomes prepared from equimolar mixtures of synthetic phosphatidylethanolamine (PE) and phosphatidylcholine (PC) with *cis* mono-unsaturated acyl chains. The activity of the branched-chain amino acid carrier is determined by the bulk properties of the membrane (Driessen, A.J.M., Zheng, T., In 't Veld, G., Op den Kamp, J.A.F. and Konings, W.N. (1988) Biochemistry 27, 865–872). PE acts as an activator and PC is ineffective. Counterflow and protonmotive-force driven transport of leucine is sensitive to changes in the acyl chain carbon number of both phospholipids and maximal with dioleoyl-PE / dioleoyl-PC. Above the gel to liquid-crystalline phase transition temperature of the lipid species, membrane fluidity decreased with increasing acyl chain carbon number. Our data suggest that the carbon number of the acyl chains of PE and PC determine to a large extent the activity of the transport system. This might be relevant for the interaction of PE with the transport protein. Variations in the acyl chain composition of PC exert a more general effect on transport activity. The acyl chain composition of phospholipids determines the membrane thickness (Lewis, B.A. and Engelman, D.M. (1983) J. Mol. Biol. 166, 211–217). We therefore propose that the degree of matching between the lipid-bilayer and the hydrophobic thickness of the branched-chain amino acid carrier is an important parameter in lipid–protein interactions.

Introduction

Biological membranes are composed of approx. 50% lipids and 50% proteins (by weight). The lipid composition strongly depends on the membrane species and can vary with respect to the headgroup, chain length, and degree of unsaturation [1]. Major lipid species of the cytoplasmic membrane of the Gram-positive bacteria *Lactococcus lactis* are phosphatidylglycerol, cardiolipin, glycolipids and phosphoglycolipids [2,3].

Membrane proteins can be either extrinsic or intrinsic. Bacterial transport proteins, belonging to the second class of membrane proteins, are integrated into the bilayer. The transmembrane portions of these proteins consist predominantly of hydrophobic amino acids, making the transmembrane segment compatible with the hydrophobic interior of the lipid bilayer. These hydrophobic transmembrane regions are adjoined by polypeptide domains with a relatively high incidence of charged amino acids. Transmembrane proteins are firmly locked in their position in the lipid bilayer by the hydrophobic effect. Charged regions cannot enter the hydrophobic interior of the membrane and the hydrophobic portion of the protein are incompatible with

Abbreviations: Bca, branched chain amino acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; di(14:1)PC, dimyristoleoyl-PC; di(16:1)PC, dipalmitoleoyl-PC; di(18:1)PC, dioleoyl-PC; di(20:1)PC, dierusenoicyl-PC; di(22:1)PC, dierucoyl-PC; $\Delta\bar{\mu}_{H^+}$ electrochemical gradient of protons; R_{18}, octadecyl rhodamine-B chloride; TPP^+, tetraphenylphosphonium ion; TMA-DPH, 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene; $\Delta\psi$, transmembrane electrical potential; ΔpH, transmembrane pH gradient.

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water. Therefore, it can be expected that membrane protein activity is modulated by both the headgroup and the fatty acid acyl chains of membrane lipids [4].

The transport system for branched-chain amino acids (*Bca* carrier) of the *L. lactis* catalyzes the uptake of L-leucine, L-isoleucine and L-valine in symport with one proton [5–7]. To study the interaction of the *Bca* carrier with the lipid environment, membrane vesicles derived from *L. lactis* have been fused with liposomes with a defined (phospho-)lipid composition by a freeze/thaw-sonication method. By this procedure membrane vesicles are enriched up to 95% by exogenous (phospho-)lipid [8–10]. The freeze/thaw-sonication procedure exhibits little phospholipid specificity, and closed hybrid membranes which retain energy-conserving properties can be formed with liposomes of various composition. This approach has been taken to investigate systematically the role of the phospholipid head group [3] and the effect of cholesterol [11] on the *Bca* carrier activity of *L. lactis* subsp. *cremoris*.

Among a variety of (phospho-)lipids only aminophospholipids, such as phosphatidylethanolamine (PE) and phosphatidylserine, and glycolipids are effective in supporting leucine transport activity. Aminophospholipids are completely absent in these membranes. A common property between the activating lipid species is the ability to form hydrogen bonds. Hydrogen bond formation between the carrier and the membrane lipids may stabilize the carrier in an active conformation [3]. Such bonding has also been implicated for the interaction between membrane lipids and the lactose carrier of *Escherichia coli* [12,13] and the Na^+-dependent leucine transport system of *Pseudomonas aeruginosa* [14]. Following a similar approach, a dramatic inhibitory effect of cholesterol on the activity of the *Bca* carrier has been noted [11]. The reduction in maximal rate of leucine transport coincides with a decrease in membrane fluidity, suggesting an important role of membrane fluidity in lipid–protein interactions.

In bacteria, homeostasis of membrane fluidity is realized by modulating the number of carbons and/or degree of unsaturation of the acyl chains of membrane lipids [15,16]. Phospholipids in bacterial membranes are usually composed of a wide range of fatty acid acyl chains. In the present communication, we report on the dependence of the *Bca* carrier of *L. lactis* on the fatty acid acyl chain composition of the phospholipids. Leucine transport activity was assayed in hybrid membranes composed of mixtures of synthetic PEs and PCs with various acyl chain carbon numbers. The activity of the *Bca* carrier senses the acyl chain composition of both the PE and PC species, and is maximal in lipid mixtures composed of dioleoyl-PE (di(18:1)PE) and dioleoyl-PC (di(18:1)PC). The acyl chain carbon number determines to a large extent the thickness of the bilayer. It is suggested that membrane thickness regulates transport activity via hydrophobic matching of the membrane protein.

Methods

Bacteria, growth conditions and isolation of membrane vesicles

Lactococcus lactis subsp. *lactis* ML_3 was grown on a chemically defined medium with 0.3% (w/v) lactose at 30°C and pH 6.4 [17,18]. Membrane vesicles were obtained by osmotic lysis [19] and stored in liquid nitrogen for later use.

Lipid extraction and analysis

L. lactis cells were incubated for 30 min in the presence of 4.2 mg lysozyme/ml at 37°C [20]. Lipids were extracted as in Viitanen et al. [21], dissolved in chloroform/methanol (9:1, v/v) and stored at −20°C under N_2. Fatty acids were detected as methyl esters [22] by gas-liquid chromatography (GLC). For GLC analysis, a 3% silar 5 CP column on 80/100 gaschromosorb Q_{11} was operated at 180°C with N_2 as carrier gas.

Lipid synthesis

Dimyristoleoyl-PC [di(14:1)PC] and dierucoyl-PC [di(22:1)PC] were enzymatically converted into the corresponding PEs with phospholipase D [23,24]. PC was dissolved in diethyl ether (100 mg PC/6 ml) and added to 12 ml of a solution containing 100 mM $CaCl_2$ and 16 mM ethanolamine. This solution was adjusted to pH 5.6 with acetic acid. After 15 min incubation at 37°C, phospholipase D (15 mg) (purified from Brussels sprouts, as described by Davidson and Long [25]) was added. Incubation was continued for 30 min with vigorous stirring to allow complete mixing of both phases. Conversion was followed with TLC. With incomplete conversion of PC, additional phospholipase D (15 mg) was added to the suspension. Incubation was prolonged until only trace amounts of PC remained. The reaction was terminated by the addition of 200 mM EDTA and adjustment of the pH to 8.0 using 2 M NaOH. Diethyl ether was removed by vacuum evaporation, and lipids were extracted as described [26]. Phosphatidic acid and traces unreacted PC were removed by silica gel column chromatography (Kiesel gel 60, 70–230 mesh, 35 × 2.5 cm). PE was eluted with chloroform/methanol/water (65:35:2, v/v). The overall yield of PE was about 50%.

Liposome formation and membrane fusion

PE and PC were mixed in equimolar ratio in chloroform/methanol (9:1, v/v). The mixture was dried under a stream of N_2 gas. Traces of solvent were removed under vacuum for 1 h. The dry lipid was

hydrated in 50 mM potassium phosphate (pH 7.0) to a final concentration of 26 mM of phospholipid phosphorous. Lipids were dispersed by ultrasonication using a bath sonicator (Sonicor, Sonicor Instruments NY). Liposomes were obtained by sonication (probe-type; MSE Scientific Instruments, West Sussex, U.K.) for 300 s at an intensity of 4 μm (peak-to-peak). Intervals of 15 s sonication and 45 s rest were used, and sonication was performed at 4°C under a constant stream of N_2.

Fusion of liposomes with *L. lactis* membrane vesicles was performed by freeze/thaw-sonication as described previously [3,8,10]. *L. lactis* membrane vesicles (0.75 mg protein) and liposomes (9.75 μmol of phospholipid) were mixed in a final volume of about 500 μl 50 mM potassium phosphate (pH 7.0). The suspension was rapidly frozen into liquid N_2 and stored until use. Frozen membranes were slowly thawed at room temperature and sonicated for 8 s with a probe sonicator.

Transport assays

$\Delta\tilde{\mu}_{H^+}$ driven L-leucine transport was performed essentially as described [3]. Hybrid membranes were incubated for 1 h at 25°C in a solution containing 20 mM potassium phosphate (pH 7.0) and with 100 mM potassium acetate. Valinomycin was added to a concentration of 2 nmol/mg of protein. Membranes were concentrated by centrifugation for 45 min at 53000 rpm ($210000 \times g_{max}$) in a Beckman type 75 Ti rotor at 5°C. Samples of 2 μl of the concentrated membrane suspension (approx. 7.5 mg of protein/ml) were diluted into 200 μl solution containing 20 mM sodium phosphate (pH 7.0), 100 mM piperazine-*N*,*N*'-bis(2-ethanesulfonate) (Pipes) and 1.5 μM L-[U-^{14}C]leucine. At different times, the reaction was arrested by dilution into 2 ml ice-cold 0.1 M LiCl. The sample was collected on a 0.45 μm cellulose nitrate filter (Millipore). The filter was washed once with 2 ml ice-cold 0.1 M LiCl, and the radioactivity was determined by liquid scintillation spectrophotometry.

For L-leucine counterflow, the hybrid membranes were incubated for 1 h at 25°C in a buffer containing 50 mM potassium phosphate (pH 7.0), and 5 mM L-leucine. Valinomycin and nigericin were added to a final concentration of 1 nmol/mg of protein. Loaded membranes were collected as described above. Samples of 2 μl (approx. 9.5 mg protein/ml) were diluted into 200 μl of 50 mM potassium phosphate (pH 7.0), containing 1.5 μM L-[U-^{14}C]leucine. The initial rate of counterflow (exchange) was determined after 10 s. Initial rates were determined at least in 4-fold. Values were normalized on the basis of the protein content.

Determination of the electrical potential

The transmembrane electrical potential ($\Delta\psi$, interior negative) was estimated from the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) using a TPP^+-selective electrode [27]. For $\Delta\psi$ measurements, 6 μl of a concentrated membrane suspension used for imposed $\Delta\tilde{\mu}_{H^+}$-driven leucine uptake was diluted into 600 μl of a buffer containing 20 mM sodium phosphate (pH 7.0), 100 mM sodium acetate, 3.3 μM TPP^+, and 5 mM $MgSO_4$. $\Delta\psi$ was dissipated by the addition of 0.2 μM nigericin.

Fluorescence polarization measurements

DPH (1,6-diphenyl-1,3,5-hexatriene) and TMA-DPH (1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene) polarization measurements were carried out essentially as described [28]. For (TMA-)DPH labelling of the hybrid membranes, 5 μl of (TMA-)DPH stock solution (4 mM in dimethylsulfoxide) was diluted into 245 μl of 50 mM potassium phosphate (pH 7.0) and vigorously mixed. Samples were taken from this suspension and added to an equal volume of hybrid membrane suspension, yielding a (TMA-)DPH to lipid molar ratio of about 1 to 250. The mixture was slowly shaken at room temperature for 1 h. Labelled membranes (100 μl) were added to 2 ml of 50 mM potassium phosphate, (pH 7.0) and the steady-state Fluorescence polarization (r_{ss}) was measured with a Perkin-Elmer LS-50 spectrofluorometer at 25°C. Excitation was at 360 nm. The fluorescence intensity at 430 nm was measured parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the emitted light. Eqn. 1 was used to calculate r_{ss} [28–30] from the corrected fluorescence intensities.

$$r_{ss} = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp) \quad (1)$$

Electron microscopy

For freeze-fracture electron microscopy, a concentrated suspension of membranes in 30% (v/v) glycerol was rapidly frozen into a mixture of solid and liquid N_2. Freeze-etch replicas were prepared by the method of Deamer et al. [31] with a Balzer BA 360 freeze etching unit.

Other analytical procedures

The fusion efficacy was determined with the R_{18} (octadecyl rhodamine-B chloride) fusion assay [32] as described previously [3]. The trapped volume of fused membranes was determined with the fluorophore calcein [3,10,11,33]. Membranes were fused in the presence of 100 μM calcein and the fluorescence was determined before and after the addition of 100 μM $CoCl_2$ (excitation, 480 nm; emission, 520 nm). The residual fluorescence after the addition of 1% (v/v) Triton X-100 was subtracted. The entrapment efficacy of 0.45 μm cellulose nitrate filters used in the transport assay was calculated from the total volume of calcein trapped by the hybrid membranes and the

volume that passed through the filter [11]. Protein was determined by the method of Lowry et al. [34] in the presence of 0.5% (v/v) sodium dodecylsulfate [35]. Bovine serum albumin was used as a standard. The concentration of the liposome preparations was determined by phosphate analysis [36].

Materials

Synthetic phospholipids were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). All lipids were checked for purity with thin-layer chromatography (TLC). R_{18}, DPH, and TMA-DPH were obtained from Molecular Probes, Inc. (Eugene, OR). L-[U-^{14}C]Leucine (12.4 TBq/mol) was obtained from New England (Dreieich, F.R.G.).

Results

Lipid acyl chain composition

The acyl chain composition of a total membrane lipid extract of *L. lactis* membrane vesicles is shown in Table I. Predominant fatty acids are 16:0, 18:1 and lactobacillic acid (19 ∇). The lipid acyl chain composition is very similar to that reported for other *Lactococcus* species [37]. The average acyl chain carbon number is 17.1, while the mean number of unsaturated groups per acyl chain ($\Sigma_{unsat}/\Sigma_{total}$) is about 0.42.

Effect of the acyl chain carbon number on $\Delta\tilde{\mu}_H$-driven leucine uptake

The branched-chain amino acid (*Bca*) transport system of *L. lactis* exhibits a high activity when reconstituted into mixtures of PE and PC [3]. A wide range of synthetic PCs is commercial available. This allows a detailed investigation of the role of the lipid acyl chain composition on the activity of the *Bca* carrier. Liposomes were prepared from equimolar mixtures of various synthetic *cis* mono-unsaturated PCs and PEs with defined acyl chain carbon number (n). The gel to liquid-crystalline phase transition temperatures of these lipid species are well below 25°C. All measurements were performed with the phospholipids in the liquid-crystalline state. *L. lactis* membrane vesicles were fused with di(18:1)PE/di(n:1)PC (1:1, mol/mol) liposomes by the freeze/thaw-sonication procedure. The acyl chain carbon number of PC was varied (n = 14, 16, 18, 20 or 22). For imposed $\Delta\tilde{\mu}_H$-driven leucine uptake experiments, hybrid membranes were equilibrated in a buffer containing 20 mM potassium phosphate (pH 7.0), and 100 mM potassium acetate in the presence of the ionophore valinomycin. Loaded membranes were rapidly diluted into a solution containing 20 mM sodium phosphate (pH 7.0), and 100 mM sodium Pipes. This procedure establishes both a pH gradient (ΔpH) and a membrane potential ($\Delta\psi$) across the membrane. All membrane preparations exhibited a transient uptake of leucine (Fig. 1). The initial rate of leucine uptake varied with the acyl chain carbon number of the PC, and was maximal with di(18:1)PC and di(16:1)PC. Observed variations in rate of leucine uptake may be a direct consequence of the lipid composition. However, differences in the magnitude of the generated $\Delta\tilde{\mu}_{H^+}$ as a result of an altered ion-permeability of the membrane may cause similar effects. The ability to sustain an imposed $\Delta\tilde{\mu}_{H^+}$ was tested by direct measurements of the $\Delta\psi$ with an ion-selective electrode which monitors the external concentration of the lipophilic cation tetraphenylphosphonium (TPP^+) (Fig. 2). In all cases, a transient $\Delta\psi$ was generated which collapsed upon the addition of the ionophore nigericin. Both the uptake and release of TPP^+ varied markedly with the acyl chain composition. The permeability of the membranes for TPP^+ decreased with increasing acyl chain carbon number of the di(n:1)PC species (Fig. 2). This aspect seriously complicates the quantitative assessment of the magnitude of the transiently imposed $\Delta\psi$. By this method, it is not possible to make a distinction between a direct modulating effect of the acyl chain composition on the activity of the *Bca* carrier and changes affected by unrelated physical properties of the membranes, such as ion-permeability.

Effect of the acyl chain carbon number on counterflow uptake of leucine

The effect of the lipid acyl chain composition on leucine transport was further studied with the counterflow technique. Unlike $\Delta\tilde{\mu}_H$-driven uptake of leucine, the carrier remains protonated under conditions of counterflow [6]. Under those conditions, transport of

TABLE I

Lipid acyl chain composition of L. lactis membranes

Cells were harvested in the late logarithmic growth phase and membrane lipids were extracted as described under Materials and Methods. ∇ designates cyclopropane containing acyl chain. N.I., not identified.

Acyl chain carbon number and number of double bonds	%
14:0	7.9
16:0	31.3
16:1	1.8
16:2	8.2
18:0	3.7
18:1	16.6
20:2	2.6
22:1	1.6
19∇	20.6
N.I.	5.7
% unsaturation	42
Average acyl chain carbon number	17.1

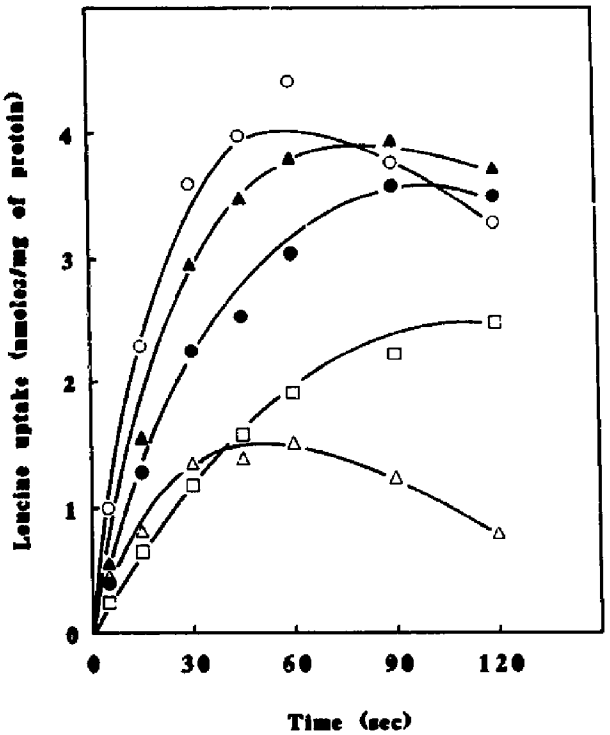

Fig. 1. $\Delta\tilde{\mu}_{H^+}$-driven leucine transport in hybrid membranes obtained by fusion of *L. lactis* membrane vesicles with liposomes composed of di(18:1)PE and di(*n*:1)PC. Leucine uptake upon the imposition of a $\Delta\tilde{\mu}_{H^+}$ was measured in hybrid membranes composed of di(18:1)PE-/di(14:1)PC (△); di(18:1)PE/di(16:1)PC (○); di(18:1)PE/di(18:1)PC (▲); di(18:1)PE/di(20:1)PC (●); and di(18:1)PE/di(22:1)PC (□). The molar lipid composition (PE/PC) of the liposomes was 1:1. *n* represents the number of C-atoms of the lipid acyl chain.

leucine can be studied in the absence of a $\Delta\tilde{\mu}_{H^+}$. Since the rate of leucine uptake is not affected by the ion-permeability of the membrane, the counterflow activity provides a more accurate assessment of *Bca* carrier activity. Lipid-enriched membranes were loaded with 5 mM leucine and an outwardly-directed leucine concentration gradient was imposed by diluting the vesicles 100-fold into a solution containing 1.5 μM [^{14}C]leucine. A transient accumulation of [^{14}C]leucine (i.e. overshoot) is observed (not shown). This overshoot phenomenon is caused by two competing processes, a rapid exchange and slow efflux reaction. At the pH measured, exchange is faster than efflux [6]. The initial rate of [^{14}C]leucine uptake can, therefore, be taken as a direct measure for transport activity. The initial rate of leucine uptake was estimated from the amount of label accumulated during the first 10 s. Leucine counterflow activity by hybrid membranes composed of di(18:1)PE/di(*n*:1)PC was a function of the acyl chain carbon number in di(*n*:1)PC (Fig. 3). Activity decreases in the following order: $n = 18 = 16 \gg 20 > 22 > 14$. These results suggest that the activity of the *Bca* carrier is modulated by the acyl chain carbon number of PC in binary mixtures of PE and PC.

Effect of the acyl chain carbon number on the steady state fluorescence polarization of DPH and TMA-DPH

Variations in leucine counterflow activity with acyl chain carbon number of PC are possibly caused by corresponding changes in membrane fluidity. The steady-state fluorescence polarization (r_{ss}) of 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) was measured for hybrid membranes composed of di(18:1)PE/di(*n*:1)PC. The hydrophilic group of TMA-DPH anchors the molecule at the headgroup region of the bilayer with the DPH moiety aligned with the phospholipid acyl chain. TMA-DPH, therefore, functions as a reliable reporter of the overall fluidity in the hydrophobic core region of the membrane [38]. As reported by others [28], an increase in chain carbon number results in a parallel change in the r_{ss} value of both DPH and TMA-DPH (Fig. 4). With increasing acyl chain carbon number, the membrane order increased resulting in a lowering of the membrane fluidity. With all lipid mixtures, fluorescence measurements were performed at a temperature beyond their gel to liquid-crystalline phase transition temperature. The

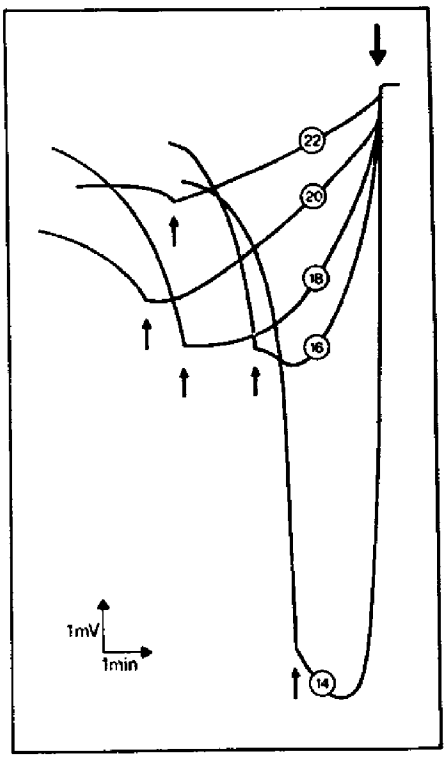

Fig. 2. Time-course of tetraphenylphosphonium ion uptake by hybrid membranes composed of di(18:1)PE and di(*n*:1)PC. The arrow indicates the addition of hybrid membranes in the presence of valinomycin (2 nmol/mg of protein); the small arrow marks the addition of 0.2 μM nigericin to dissipate $\Delta\psi$.

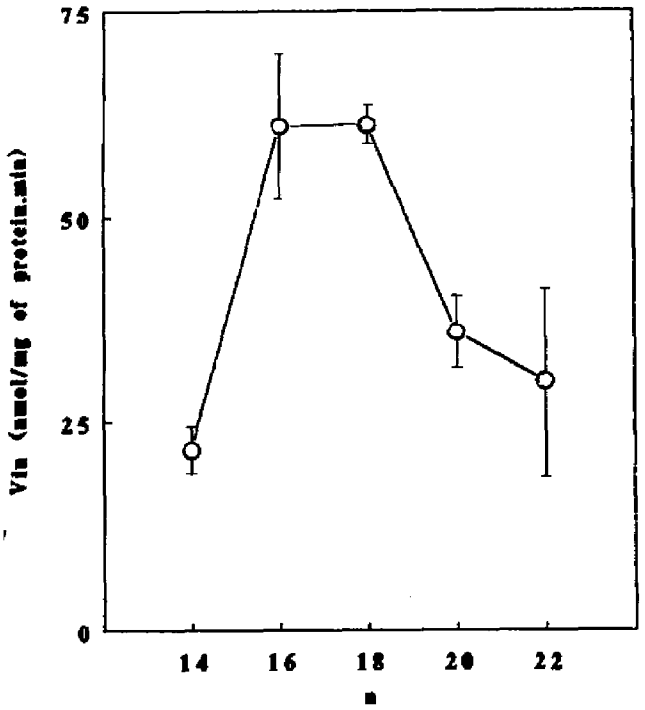

Fig. 3. Initial rates of counterflow uptake of leucine in hybrid membranes obtained by fusion of *L. lactis* membrane vesicles with liposomes composed of di(18:1)PE/di(*n*:1)PC. The initial rate of leucine uptake was calculated from the amount of [^{14}C]leucine accumulated within 10 s. Each result is the mean of multiple estimates ($n = 4$) with shown ± S.E.

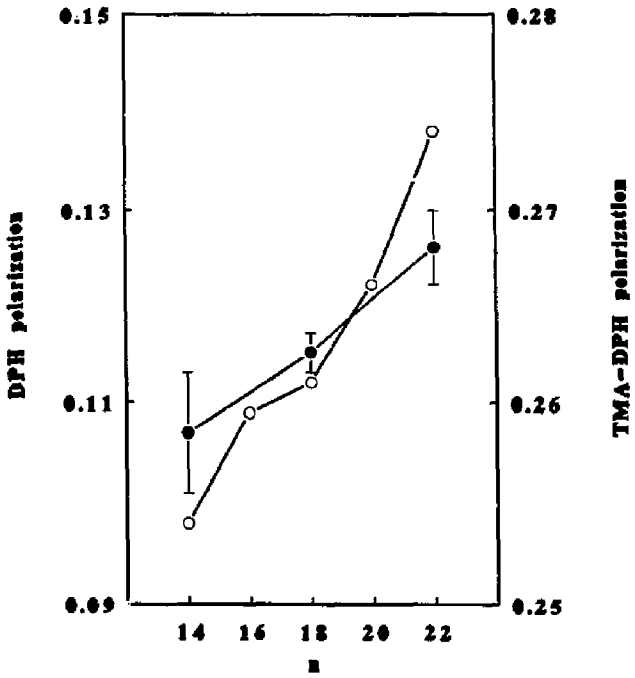

Fig. 4. DPH (○) and TMA-DPH (●) anisotropy (r_{ss}) in hybrid membranes obtained by fusion of *L. lactis* membrane vesicles with liposomes composed of di(18:1)PE/di(*n*:1)PC.

relative differences in membrane fluidity among these membranes were small compared to the change induced by a gel to liquid-crystalline phase transition (not shown).

Effect of the acyl chain carbon number in hybrid membranes composed of di(n:1)PE/di(n:1)PC

In the experiments described above, the acyl chain carbon number of PC was varied while the composition of PE remained constant. Possible artifacts may arise from defects in the bilayer membrane structure as a consequence of the immiscibility of lipids with different acyl chain carbon number. To exclude this possibility, liposomes were prepared with an equimolar mixture of PE and PC with identical acyl chain carbon number. Only a limited number of *cis* mono-unsaturated PEs are commercially available. Therefore, di(14:1)PE and di(22:1)PE were prepared by an enzymatic reaction from the corresponding PCs. *L. lactis* membrane vesicles were fused with di(*n*:1)PE/di(*n*:1)PC liposomes with *n* equal to 14, 18, or 22. As with the hybrid membranes containing di(18:1)PE/di(*n*:1)PC, maximal counterflow activity was observed when the acyl chain carbon number equaled 18 (Table II). The r_{ss} for DPH increased with acyl chain carbon number (Table II).

PE is the stimulating lipid component in mixtures of PE and PC [3]. To define the role of PE in more detail, liposomes were made from di(14:1)PE/di(22:1)PC and di(22:1)PE/di(14:1)PC and fused with membrane vesicles. The counterflow activities in the hybrid membranes composed of di(14:1)PE/di(22:1)PC and di(22:1)PE/di(14:1)PC correspond closely to those found with di(14:1)PE/di(14:1)PC and di(22:1)PE/di(22:1)PC, respectively (Table II). These results further support the finding that the acyl chain composition of PE is an important determinant of the activity of the *Bca* carrier. The 'average' acyl chain carbon number of the lipid species is of less importance. r_{ss} values of hybrid membranes composed of di(14:1)PE/

TABLE II

DPH polarization and initial rate of counterflow of leucine by hybrid membranes composed of membrane vesicles of L. lactis fused with liposomes containing PE/PC (1:1, mol/mol) with different acyl chain carbon numbers

Composition of liposomes fused with *L. lactis* [a]	Leucine uptake rate (nmol/mg of protein per min)	DPH polarization (r_{ss})
di(14:1)PE/di(14:1)PC	14.9	0.089
di(18:1)PE/di(18:1)PC	27.7	0.095
di(22:1)PE/di(22:1)PC	9.1	0.173
di(14:1)PE/di(22:1)PC	14.3	0.131
di(22:1)PE/di(14:1)PC	7.9	0.112

[a] Molar lipid composition (PE/PC) was 1:1.

TABLE III

Physical properties of hybrid membranes

Composition of liposomes fused with *L. lactis* [a]	Fusion efficacy [b] (%)	Calcein trapping volume [c] (μl/mg of protein)	Filter entrapment efficacy [d] (%)
di(18:1)PE/di(14:1)PC	88.4	23.5	95.6
di(18:1)PE/di(22:1)PC	84.0	17.3	98.4
di(14:1)PE/di(14:1)PC	85.3	16.8	99.8
di(18:1)PE/di(18:1)PC	84.7	23.6	99.9
di(22:1)PE/di(22:1)PC	81.1	20.3	99.8
di(14:1)PE/di(22:1)PC	85.3	17.1	99.8
di(22:1)PE/di(14:1)PC	79.0	10.9	99.8

[a] Molar lipid composition (PE/PC) was 1:1.
[b] Fusion efficacy was determined using the R_{18} fusion assay.
[c] Trapping volume of fused membranes was estimated from the amount of internal calcein.
[d] Filter entrapment efficacy was determined from the recovery of the trapped amount of calcein after filtration.

di(22:1)PC and di(22:1)PE/di(14:1)PC are comparable (Table II). Since there is no detectable preference of the DPH molecules for either lipid mixture, an interference by phospholipid phase separations is unlikely.

Physical properties of hybrid membranes

A number of physical properties of the hybrid membranes are shown in Table III. For each lipid composition tested, hardly any difference in internal volume, fusion- and filter entrapment efficacy was observed. These comparable structural characteristics were also evident from freeze-fracture electron microscopy studies. The electron microscope images of hybrid membranes obtained with di(14:1)PE/di(14:1)PC (Fig. 5A), di(18:1)PE/di(18:1)PC (Fig. 5B) and di(22:1)PE/di(14:1)PC (Fig. 5C) liposomes showed a similar distribution of both size and intramembranous particle distribution. In all cases, closed membrane structures were observed. The average diameter varies from 50 to 100 nm. Fusion was also evident from these images. The immense dilution of membrane proteins by the incorporation of exogenous phospholipid results in a low intramembranous particle density compared to the original non-fused bacterial membranes (not shown).

Discussion

The acyl chain dependency of the transport system for branched-chain amino acids (*Bca*) in *Lactococcus lactis* was studied in hybrid membranes obtained by fusion of membrane vesicles of *L. lactis* with PE/PC liposomes [3,9–11] containing different fatty acid acyl chain compositions. The freeze/thaw-sonication technique was employed since this method does not involve the use of detergents, and exhibits little phospholipid specificity (Table III) [3,39]. The lipid composition of the fused membranes can thus be changed in a convenient and controlled manner, while the number of transport systems in the bilayer is kept constant.

The *Bca* carrier exhibits a high activity when reconstituted in mixtures of PC and PE [3]. Activity increases with the PE content of the liposomes. In the present study, a mixture of PE/PC (1:1, mol/mol) was used which allow the study of bulk effects exerted by changes in fatty acid acyl chain composition of both lipid species. Oleic acid (18:1) is an important constituent of a membrane lipid extract of *L. lactis* (Table I). Therefore, liposomes were used composed of di(18:1)PE/di(*n*:1)PC (1:1, mol/mol). *n* represents the number of carbon atoms in the acyl chain.

Transport activity measurements assayed by $\Delta\tilde{\mu}_H$-driven leucine uptake and leucine counterflow yield similar results. The permeation rate of the lipophilic cation TPP^+ across the lipid bilayer was strongly de-

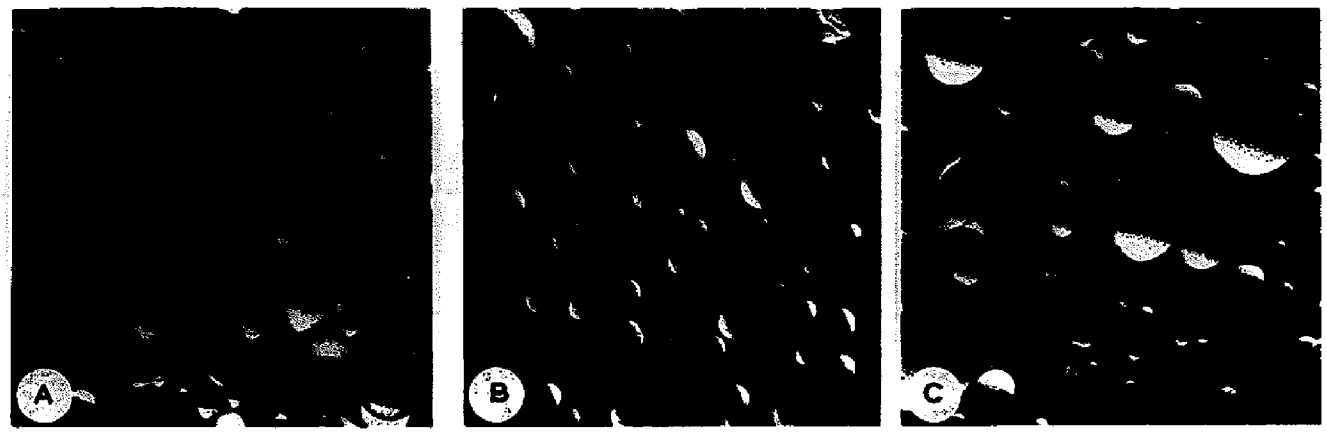

Fig. 5. Freeze-fracture replicas of hybrid membranes obtained by fusion of *L. lactis* membrane vesicles fused with liposomes composed of di(14:1)PE/di(14:1)PC (A), di(18:1)PE/di(18:1)PC (B) and di(14:1)PE/di(22:1)PC (C). Bar = 0.25 μm.

pendent on the acyl chain composition. The restricted TPP^+ permeation rate correlates with an increase in acyl-chain ordering, and may thus directly relate to membrane fluidity. Nakazato et al. [40] measured the TPP^+ permeation in liposomes composed of egg-yolk PC with various amounts of cholesterol. The permeability of TPP^+ decreased with cholesterol content of the liposomes. A tentative correlation between the permeability and membrane fluidity was proposed. It is concluded that under non steady-state conditions, the transmembrane distribution of TPP^+ cannot be used to measure $\Delta\psi$. Alternatively, the initial counterflow activity was used to assess transport activity. The initial rate of leucine counterflow is essentially determined by a rapid $\Delta\bar{\mu}_{H^+}$-independent exchange reaction [6]. High rates of counterflow were found with di(18:1)PE/di(n:1)PC membranes in which n equaled 18 and 16 (Fig. 3). Although PE is the active component in these mixtures [3], variations in acyl chain carbon number of PC caused marked changes in the transport activity. When the acyl chain carbon numbers of PC diverged from 16 or 18 a low transport activity is noted. Maximal activity is observed with an acyl chain carbon number that resembles that of the most prominent fatty acid species in natural *L. lactis* membranes (Table I). Similar results were obtained with di(n:1)PE/di(n:1)PC membranes (Table II) and maximal activity was found with $n = 18$. Uratani et al. [41] studied the effect of the fatty acid acyl chain composition on the Na^+-dependent leucine transport system of the Gram-negative bacterium *Pseudomonas aeruginosa*. It was concluded that the activity of the carrier is defined by the 'average acyl chain length' of the lipid species present. Maximal transport activity was observed with an average acyl chain carbon number of 17. This value approaches the mean value of 16.9 found for *P. aeruginosa* membrane lipids. Our results, and those of others [42] suggest that the term 'average chain length' is not a very useful qualification of acyl chain composition. Membranes composed of equimolar mixtures of di(14:1)PE/di(22:1)PC, di(22:1)E/di(14:1)PC or di(18:1)PE/di(18:1)PC, which give similar average acyl chain carbon numbers (i.e. 18), support activity to different extent (Table II).

In a previous study [11] we showed that the leucine transport in PE/PC (3:1, mol/mol) membranes is suppressed by cholesterol. With increasing cholesterol content, the relative membrane fluidity decreased as deduced from the increase in the r_{ss} value for the intrinsic membrane probe DPH. The impact of cholesterol was correlated to its effect on the fluidity of the membrane. With di(18:1)PE/di(n:1)PC membranes, the r_{ss} values for (TMA-)DPH were found to increase with acyl chain carbon number (Fig. 4). However, a progressive correlation between the transport activity and membrane fluidity is not observed. Optimal transport activity appears to relate to intermediate membrane fluidity values, above which, activity declines. Comparable results have been reported for the Ca^{2+}-Mg^{2+}-dependent ATPase of sarcoplasmic reticulum [43], the mammalian erythrocyte glucose facilitator [44,45] reconstituted into PCs with different acyl chain composition, and the Na^+-coupled leucine transport system of *P. aeruginosa* [41]. Three possible explanations have to be considered. First, transport activity relates to membrane fluidity, but requires an optimal balance between these two parameters. Secondly, transport activity increases with increasing membrane fluidity. In the liquid-crystalline state other physical properties of the membrane may affect transport as well. Third, transport activity is not determined by membrane fluidity at all but by other physical properties of the membrane. At a qualitative level, there is no obvious relationship between membrane fluidity and transport activity. Our observation that the acyl chain carbon number of the lipid species modulate transport activity can not be explained by the effect on membrane fluidity only. A simpler physical interpretation of these results may be found in the hydrophobic membrane thickness. Quantitative information on the hydrophobic membrane thickness of phospholipid mixtures is scarce [46–50], there is an increasing amount of evidence that such properties modulate lipid-protein interactions in membranes [50–52] and regulate a variety of membrane functions [53]. The acyl chain carbon number is a major determinant of the thickness of the bilayer [46–50]. The differences in membrane thickness caused by the variation in acyl chain carbon number can be large. For instance, low-angle X-ray diffraction studies of liposomes composed of *cis* mono-unsaturated PCs indicate that the membrane thickness increases from 3.6 nm to 5.3 nm when the acyl chain carbon number is varied between 12 and 24 [50]. Other factors such as the unsaturation level of the acyl chains and the order of the lipids in the membrane may contribute as well [46–50]. The degree of matching between the hydrophobic thickness of an integral membrane protein and the lipid-bilayer may be essential for the stabilization of an active conformation of the protein. As schematically depicted in Fig. 6, the protein may have to adjust the bilayer thickness by changing its conformation. Alternatively, the integral membrane protein may span the membrane by locally deforming the lipid structure when the hydrophobic bilayer thickness is not optimal. This process may involve a local reduction of membrane thickness by interdigitation of the phospholipid acyl chains. Yet, the exact nature of the mechanism cannot be specified.

In the present study, optimal activity was observed when the acyl chain carbon number resembles that of the major fatty acid components in natural membranes. Small changes in the bulk properties of the membrane,

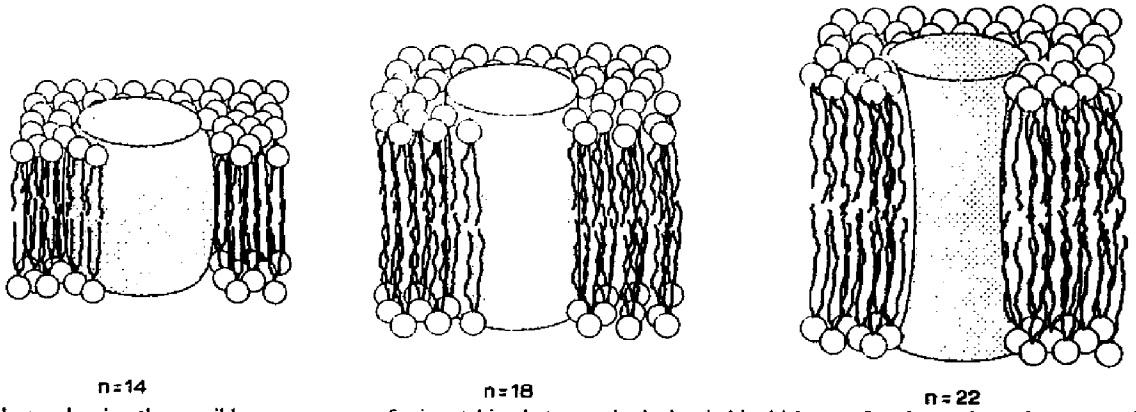

Fig. 6. Scheme showing the possible consequence of mismatching between the hydrophobic thickness of an integral membrane protein and the membrane. The integral membrane protein responds to the membrane thickness by changing its conformation. Phospholipid molecules and membrane proteins are not drawn in scale, neither are the phospholipids with different acyl chain carbon number.

i.e. acyl chain carbon number, have a profound influence on the activity of the *Bca* carrier. Although the molecular interactions will be more complex, the thickness of the membrane should obey certain boundaries to allow optimal functioning of the transport protein. An important role of membrane fluidity can not be dismissed, although minor compared to gross changes due to membrane thickness. By virtue of its effect on lipid ordering, cholesterol may affect transport activity indirectly by changing the membrane hydrophobic thickness [54,56].

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