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Effect of the unsaturation of phospholipid acyl chains on leucine transport of *Lactococcus lactis* and membrane permeability

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The effect of the degree of unsaturation of the phospholipid acyl chains on the branched-chain amino acid transport system of *Lactococcus lactis* was investigated by the use of a membrane fusion technique. Transport activity was analyzed in hybrid membranes composed of equimolar mixtures of synthetic unsaturated phosphatidylethanolamine (PE) and phosphatidylcholine (PC) in which the number of *cis* double bonds in the 18-carbon acyl chains was varied. The accumulation level and initial rate of both counterflow and protonmotive-force driven transport of leucine decreased with increasing number of double bonds. The reduction in transport activity with increasing number of double bonds correlated with an increase in the passive permeability of the membranes to leucine. The membrane fluidity was hardly affected by the double bond content. It is concluded that the degree of lipid acyl chain unsaturation is a minor determinant of the activity of the branched chain amino acid transport system, but effects strongly the passive permeability of the membrane.

Introduction

Biological membranes consist of a bilayer of lipid molecules with their polar headgroups oriented toward the aqueous phase and their hydrophobic hydrocarbon chains forming the interior of the membrane. Lipids vary with respect to the structure of the polar headgroups and the nature of the fatty acids. These fatty acids can differ in a number of aspects such as the lengths of their hydrocarbon chains and number and position of double bonds. An important feature of biological membranes is that their structure is predominantly held together by noncovalent bonds such as Van der Waals and coulombic interactions, which make them highly impermeable to small ions. This low ion-permeability of membranes results from the high energy requirement for transfer of an ion from the aqueous phase into the apolar, hydrocarbon-like interior of the membrane. This barrier is important for the functioning of the cell membrane in processes such as energy-transduction. Specific membrane proteins allow the selective passage of certain types of ions and enables the cell membrane to regulate the ionic permeability [1]. Membrane proteins can be integrated in the lipid bilayer and span the entire width of the membrane. 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. The activity of membrane proteins can be modulated by both the headgroup and the fatty acid acyl chains of membrane lipids [2].

The transport system for branched-chain amino acids (*Bca* carrier) of *Lactococcus lactis* catalyzes the uptake of L-leucine, L-isoleucine and L-valine in symport with one proton [3–5]. For the studies on 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 can be enriched up to 95% by exogenous (phospho-)lipid. The freeze/thaw-sonication procedure exhibits little phospholipid specificity and closed hybrid membranes which retain energy-con-

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Abbreviations: Bca, branched chain amino acid; DEPC, diethyl pyrocarbonate; DPH, 1,6-diphenyl-1,3,5-hexatriene; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; di(18:1)PC (or PE), dioleoyl-PC (or PE); di(18:2)PC (or PE), dilinoleoyl-PC (or PE); di(18:3)PC (or PE), dilinolenoyl-PC (or PE); $\Delta\tilde{\mu}_{H^+}$, electrochemical gradient of protons; R_{18}, octadecyl rhodamine-B chloride; TPP^+, tetraphenylphosphonium ion; TMA-DPH, 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene; $\Delta\psi$, transmembrane electrical potential; ΔpH, transmembrane pH gradient.

serving properties can be formed with liposomes of various composition [6–8]. Following this approach the role of the phospholipid headgroup [9], the fatty acid acyl chain carbon number [10], and cholesterol [11] was systematically explored. Only aminophospholipids (phosphatidylethanolamine and phosphatidylserine) and glycolipids are effective in supporting leucine transport activity. The phospholipid headgroup specificity observed are due to bulk effects. A common property among the activating lipid species is the ability to form extramolecular hydrogen bonds. Such hydrogen bonding may stabilize the carrier in an active conformation [9]. A fatty acid acyl chain number of 18 C-atoms supports optimal leucine transport activity in hybrid membrane systems. The degree of matching between the hydrophobic thickness of the transport protein and the lipid bilayer seems to be a major factor for the stabilization of the active conformation of the protein [10]. Cholesterol has an inhibitory effect on the leucine transport activity. The reduction in maximal rate of leucine transport coincides with a decrease in membrane fluidity, suggesting that membrane fluidity is an additional modulating factor in transport activity [11].

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 [12,13]. Phospholipids in bacterial membranes are usually composed of a wide range of fatty acid acyl chains, varying in the length of their hydrocarbon chains and the number and position of the *cis* double bonds. Major fatty acids identified in the natural membranes of *L. lactis* are palmitic acid (16:0), oleic acid (18:1) and the cyclopropane ring containing lactobaccilic acid ($C_{19\Delta}$). The acyl chains are highly unsaturated (up to 42%), with oleic acid as the predominant unsaturated species [10].

In the present communication we report the dependency of the *Bca* carrier of *L. lactis* on the degree of unsaturation of the fatty acid acyl chains. Leucine transport activity was assayed in hybrid membranes composed of equimolar mixtures of synthetic PE's and PC's with unsaturated acyl chains of 18 carbon atoms, which provides maximal activity. The number of double bonds of both the PE and PC species was varied. Unsaturated PE's tend to adopt the hexagonal (H_{II}) configuration. This tendency increases with increased acyl chain unsaturation. All species of phospholipids that adopt alone the bilayer phase can stabilize hexagonal-preferring lipids into an overall bilayer organization in mixed systems. The proportions of bilayer lipids, required to achieve this, can vary substantially (20–50 mol%) depending on the lipid species [14]. The increase in unsaturation of the PE and/or PC component results in an increase in permeability of lipophilic cation tetraphenylphosphonium (TPP^+), acetate and the substrate leucine, while steady-state polarization of (TMA-)DPH is not significantly affected.

Methods

Bacteria, growth conditions and isolation of membrane vesicles

Lactococcus lactis subsp. *lactis* ML_3 was grown on a chemically defined medium with 1% (w/v) galactose and 25 mM L-arginine at 30°C and pH 6.4 [15,16]. Membrane vesicles were obtained by osmotic lysis [17] and stored in liquid nitrogen for later use.

Liposome formation and membrane fusion

The formation of liposomes (9.75 μmol of phospholipid) and subsequent fusion with *L. lactis* membrane vesicles (0.75 mg protein) by freeze/thaw-sonication was performed as described [10]. Upon re-extraction of lipids from hybrid membranes and separation by two-dimensional thin-layer chromatography [9] an one-to-one molar ratio of PE/PC was found. Only traces of lipids (3–5 mol%) were present, which originated from the native cytoplasmic membrane of *L. lactis*.

Transport assays

$\Delta\tilde{\mu}_H$-driven L-leucine transport and L-leucine counterflow were performed as described [10]. Concentrated membrane suspensions contained approx. 7 mg of protein/ml for hybrid membranes. In the counterflow assay the concentration of L-[U-^{14}C]leucine was 1.5 μM for *E. coli* PL/egg PC (1:1, mol/mol) hybrid membranes and 3 μM for di(18:*m*)PE/di(18:*n*)PC (1:1, mol/mol) hybrid membranes. Transport assays with diethyl pyrocarbonate (DEPC)-treated membranes were performed after incubation of the hybrid membranes (pH 7) with different concentrations of DEPC (stock 0.7 M in ethanol) for 30 min at 25°C. DEPC was added after sonication, but before concentrating the hybrid membranes.

For L-[U-^{14}C]leucine efflux, hybrid membranes or liposomes in the presence of valinomycin and nigericin (1 nmol/mg of protein) were concentrated 4-fold by centrifugation for 45 min at 53000 rpm ($210000 \times g_{max}$) in a Beckman type 75 Ti rotor at 5°C. The concentrated membrane suspension was incubated for 1 h at 25°C in a buffer containing 50 mM potassium phosphate (pH 7.0), and 1 mM L-[U-^{14}C]leucine. Samples of 4 μl were rapidly diluted into 400 μl of 50 mM potassium phosphate (pH 7.0). The reaction was arrested by dilution into 2 ml ice-cold 0.1 M LiCl. Samples were collected on a 0.45 μM cellulose nitrate filter (Millipore), which were washed once with 2 ml ice-cold 0.1 M LiCl. Radioactivity was determined by liquid scintillation spectrophotometry. Efflux experiments were performed in triplicate and analyzed by plotting the logarithm of the intravesicular solute concentration

as a function of time. The rate of exit of L-leucine from DEPC-treated hybrid membranes and liposomes were measured. First-order rate constants (D; in min^{-1}) for passive diffusion were determined according to the following equation [3]:

$$[Leu]_t = [Leu]_0 \cdot e^{-D \cdot t} \quad (1)$$

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 as described [10].

Determination of the pH gradient

The pH gradient across the membrane (ΔpH, interior alkaline) was determined from the fluorescence of pyranine [18]. Pyranine (100 μM) was entrapped in the hybrid membranes during the sonication step. Pyranine-loaded membranes were washed with a 20-fold volume of 20 mM potassium phosphate (pH 7.0), supplemented with 100 mM potassium acetate and collected by centrifugation for 45 min at 53000 rpm ($210000 \times g_{max}$) in a Beckman type 75 Ti rotor at 5°C. Concentrated hybrid membranes (5 μl of a suspension containing about 7.5 mg of protein/ml) were diluted into 1.5 ml 20 mM potassium phosphate (pH 7.0) supplemented with 100 mM piperazine-*N*,*N*'-bis(2-ethanesulfonate) (Pipes). The internal pH was monitored by the fluorescence of pyranine (excitation, 450 nm; emission, 508 nm). The pH gradient was induced by the addition of 30 nM valinomycin and dissipated by the addition of 30 nM nigericin. A conversion factor of Z of 59 at 25°C was used to express ΔpH in millivolts.

Fluorescence polarization measurements

DPH (1,6-diphenyl-1,3,5-hexatriene) and TMA-DPH (1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene) steady-state polarization measurements were carried out as described [10]. Fluidity is used as a qualitative measure and is defined as the inverse of microviscosity. Microviscisity can be deduced from the steady-state fluorescence polarization of (TMA-)DPH probes [19].

Other analytical procedures

The fusion efficiency, the trapped volume and the entrapment efficiency was determined as described [10]. Protein was determined by the method of Lowry et al. [20] in the presence of 0.5% (v/v) sodium dodecylsulfate [21]. Bovine serum albumin was used as a standard. The concentration of the liposome preparations was determined by phosphate analysis [22].

Materials

Synthetic phospholipids were purchased from Avanti Polar Lipids, (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. (Junction City, OR). L-[U-^{14}C]Leucine (12.4 TBq/mol) was obtained from New England (Dreieich, Germany). Diethyl pyrocarbonate was obtained from Sigma Chemical (St. Louis, MO).

Results

Physical properties of hybrid membranes

The branched-chain amino acid (*Bca*) transport system of *L. lactis* exhibits a high activity when reconsti-

TABLE I

Physical properties of hybrid membranes

Composition of liposomes fused with *L. lactis* [a]	Fusion efficiency [b] (%)	Calcein trapping volume [c] (μl/mg of protein)	Filter entrapment efficiency [d] (%)	Polarization TMA-DPH (r_{ss})
Di(18:1)PE/di(18:1)PC	96.9	4.5	99.9	0.282 ± 0.003
Di(18:1)PE/di(18:2)PC	99.5	5.6	99.7	0.275 ± 0.010
Di(18:1)PE/di(18:3)PC	98.2	6.4	97.9	0.278 ± 0.005
Di(18:2)PE/di(18:1)PC	98.9	5.6	97.7	0.272 ± 0.008
Di(18:3)PE/di(18:1)PC	94.6	6.4	98.1	0.279 ± 0.007
Di(18:2)PE/di(18:2)PC	98.6	5.6	99.6	0.275 ± 0.003
Di(18:3)PE/di(18:3)PC	98.1	7.2	97.9	0.280 ± 0.011

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

tuted into mixtures of PE and PC [9,10]. The wide range of synthetic PC's and PE's commercial available allows a detailed investigation of the role of the lipid acyl chain composition on the activity of the *Bca* carrier. The effect of the phospholipid acyl chain carbon number on the activity of the *Bca* transport system of *L. lactis* has been determined previously [10]. This study focuses on the effects of the number of double bonds on the transport activity. Liposomes were prepared from equimolar mixtures of various synthetic *cis* poly-unsaturated PC's and PE's with an acyl chain carbon number of 18. The gel to liquid-crystalline phase transition temperatures of each of these lipid species is well below 25°C. The presence of 50 mol% of PC stabilizes the bilayer conformation [14]. *L. lactis* membrane vesicles were fused with di(18 : *m*)PE/di(18 : *n*)PC (1 : 1, mol/mol) liposomes by the freeze/thaw-sonication procedure. The number of double bonds in the acyl chain carbon number of PC and/or PE were varied (*m* and/or *n* = 1, 2 or 3, respectively). Physical properties of the hybrid membranes are summarized in Table I. For each lipid composition tested, closed membrane structures were obtained which do not differ significantly in fusion efficiency, internal volume, filter entrapment efficiency and membrane fluidity. The steady-state fluorescence polarization (r_{ss}) of 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) was used as a measure for membrane fluidity. DPH partitions into the hydrophobic core of lipid bilayers, whereas the amphipatic TMA-DPH molecules reside in the interfacial and headgroup region of membranes [23]. Due to its restricted mobility, TMA-DPH has been shown to be a reliable reporter of the overall fluidity in the hydrophobic core region of the membrane [24]. With all lipid mixtures, fluorescence measurements were performed at a temperature above the gel to liquid-crystalline phase transition temperature. 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). The r_{ss} values of both DPH (not shown) and TMA-DPH (Table I) were virtually similar for all lipid mixtures. These results suggest that under the experimental conditions employed, the fluid state of the unsaturated lipid species is not significantly altered by the variation in number of double bonds.

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

For the assay of artificially imposed $\Delta\tilde{\mu}_H$-driven leucine uptake, 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 transmembrane electrical potential ($\Delta\psi$) and pH gradient (ΔpH). All membrane preparations exhibited a transient uptake of leucine (Fig. 1), except for di(18:3)PE/di(18:3)PC hybrid membranes in which uptake is almost zero. The initial rate and maximal level of leucine uptake varied with the number of double bonds of the acyl chains, and was maximal with di(18 : 1)PE/di(18 : 1)PC. Though the observed variations may be a direct consequence of the lipid composition, differences in the magnitude of the generated $\Delta\tilde{\mu}_H$· as a result of an altered ion-permeability of the membrane have to be considered as well. The capacity to sustain an imposed $\Delta\tilde{\mu}_H$· was tested by direct measurements of both the $\Delta\psi$ and ΔpH (Fig. 2). $\Delta\psi$ was measured with an ion-selective electrode which monitors the external concentration of the lipophilic cation tetraphenylphosphonium (TPP^+). Changes in intravesicular pH were followed using the pH-sensitive fluorescent dye pyranine. Measurements of $\Delta\psi$ (Fig. 2A) and ΔpH (Fig. 2B) were performed for hybrid membranes composed of di(18 : *m*)PE/di(18 : *n*)PC in which *m* and *n* were varied between 1 and 3. In all cases, a transient $\Delta\psi$ (Fig. 2A) and ΔpH (Fig. 2B) was generated upon the imposition of an outwardly directed potassium and acetic acid diffusion gradient. Both $\Delta\psi$ and ΔpH rapidly collapsed upon the addition of the ionophore nigericin. The transient character of $\Delta\psi$ and ΔpH was more pronounced with

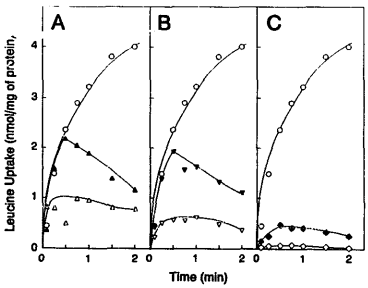

Fig. 1. $\Delta\tilde{\mu}_H$-driven leucine transport in hybrid membranes obtained by fusion of *L. lactis* membrane vesicles with liposomes composed of equimolar mixtures of di(18 : *m*)PE and di(18 : *n*)PC. (A) di(18 : 1)PE/di(18 : *n*)PC, in which $n = 1$ (○), $n = 2$ (▲), $n = 3$ (△); (B) di(18 : *m*)PE/di(18 : 1)PC in which $m = 1$ (○), $m = 2$ (▼), $m = 3$ (▽); (C) di(18 : *m*)PE/di(18 : *n*)PC in which $m = n = 1$ (○), $m = n = 2$ (◆), $m = n = 3$ (◇). *m* an *n* represent the number of double bonds of the lipid acyl chains in PE and PC, respectively. For comparison, the uptake of leucine by hybrid membranes composed of di(18 : 1)PE/di(18 : 1)PC is represented in each panel.

increasing number of double bonds. Comparable results were obtained for other combination of unsaturated PE's and PC's (not shown) and suggest that membranes bearing phospholipids with an increasing number of double bonds in the acyl chain have a lowered capacity to sustain a $\Delta\tilde{\mu}_{H^+}$. As it is not possible to obtain an accurate estimate for the magnitude of $\Delta\psi$ and ΔpH, the modulating effect of the acyl chain composition was not further defined on the $\Delta\tilde{\mu}_{H^+}$-dependent transport activity of the *Bca* carrier.

Effect of acyl chain unsaturation on counterflow uptake of leucine

The effect of the lipid acyl chain composition on leucine transport was examined with the counterflow technique. At low pH, the carrier remains protonated and exchange is favoured towards H^+-linked efflux [4]. This exchange activity does not require a $\Delta\tilde{\mu}_{H^+}$ and is not affected by the ion-permeability of the membrane. Exchange activity therefore present an accurate measure of the *Bca* carrier activity. This reaction is most conveniently measured by the counterflow technique. By this technique, exchange between extravesicular [^{14}C-]leucine and intravesicular unlabelled leucine is measured under conditions of an outwardly-directed leucine concentration gradient. Lipid-enriched membranes were loaded with 5 mM leucine and diluted 100-fold into a solution containing 3 μM [^{14}C]leucine (final concentration approx. 53 μM). A transient accumulation of [^{14}C]leucine (i.e. overshoot) is observed.

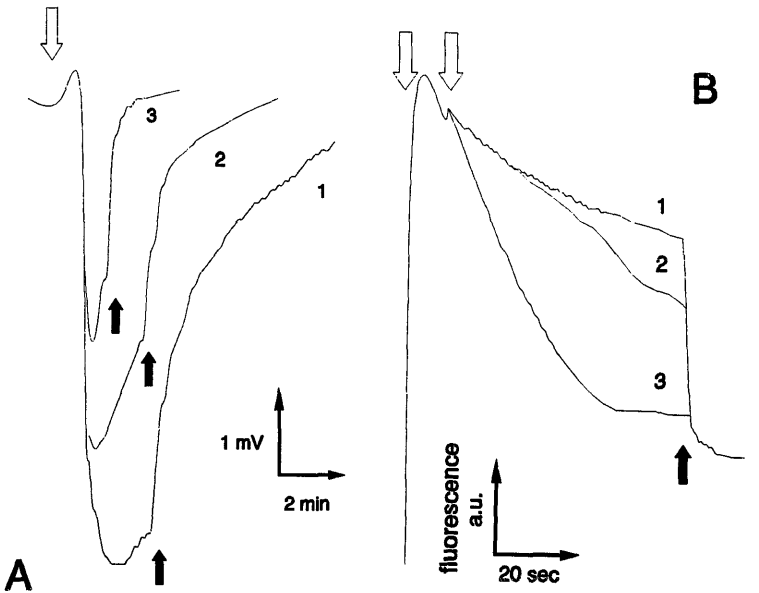

Fig. 2. Time course of tetraphenylphosphonium ion uptake (A) and pyranine fluorescence (B) by hybrid membranes composed of di(18:*m*)PE and di(18:*n*)PC, in which $m = n = 1$, 2 or 3. The open arrow indicates the addition of hybrid membranes in the presence of valinomycin (2 nmol/mg of protein) to initiate an outwardly directed potassium and acetic acid diffusion gradient. In panel B, valinomycin was added at a latter stage (second open arrow). An increase in pyranine fluorescence indicates an increase in intravesicular pH. Closed arrows mark the addition of 0.2 μM nigericin to dissipate $\Delta\psi$ and ΔpH.

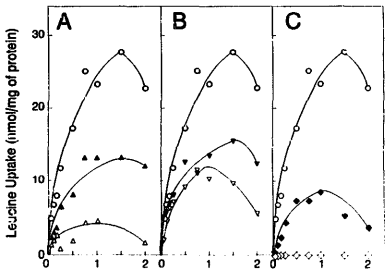

Fig. 3. Leucine counterflow uptake in hybrid membranes obtained by fusion of *L. lactis* membrane vesicles with liposomes composed of equimolar mixtures of di(18:*m*)PE and di(18:*n*)PC. (A) di(18:1)PE/di(18:*n*)PC, in which $n = 1$ (○), $n = 2$ (▲), $n = 3$ (△); (B) di(18:*m*)PE/di(18:1)PC in which $m = 1$ (○), $m = 2$ (▼), $m = 3$ (▽); (C) di(18:*m*)PE/di(18:*n*)PC in which $m = n = 1$ (○), $m = n = 2$ (◆), $m = n = 3$ (◇). *m* an *n* represent the number of double bonds of the lipid acyl chains in PE and PC, respectively. For comparison, the uptake of leucine by hybrid membranes composed of di(18:1)PE/di(18:1)PC is represented in each panel.

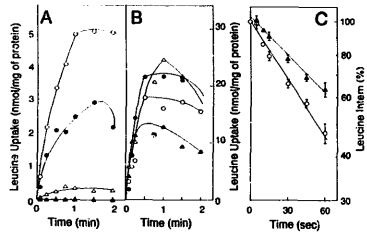

Fig. 4. $\Delta\tilde{\mu}_H$-driven leucine transport (A), leucine counterflow uptake (B) and leucine efflux (C) in hybrid membranes obtained by fusion of *L. lactis* membrane vesicles with liposomes composed of *E. coli* PL/egg PC (1:1, w/w), treated with 0 (○), 1 (●), 5 (△) and 10 mM (▲) DEPC. Error bars in panel C indicate the standard error of mean of three independent experiments.

This overshoot is caused by two competing processes, i.e. rapid exchange and slow efflux. At the pH measured, exchange is faster than efflux [4]. Leucine counterflow activity by hybrid membranes composed of di(18:*m*)PE/di(18:*n*)PC was a function of the number of double bonds (Fig. 3). Activity decreased with increasing number of double bonds. A strong reduction in activity was observed when the number of double bonds in the acyl chain of both lipid species was varied simultaneously (Fig. 3C).

Effect of acyl chain unsaturation on leucine permeability

To determine whether the hybrid membranes differ in their permeability for leucine, efflux of leucine was studied in liposomes and hybrid membranes bearing an inactivated *Bca* transport system. The *Bca* transport system was inactivated with the histidine-modifying reagent diethyl pyrocarbonate [25]. A concentration of 10 mM DEPC completely abolished $\Delta\tilde{\mu}_H$-driven up-

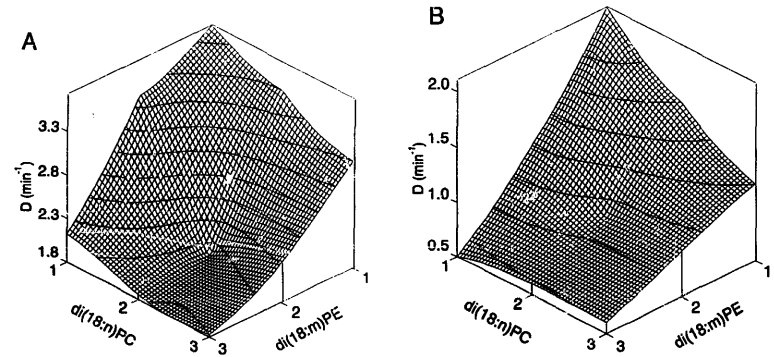

Fig. 5. Three-dimensional representation of the first-order rate constants (*D*) of leucine diffusion for liposomes (A) and DEPC-treated hybrid membranes (B) as a function of the degree of PC and PE acyl chain unsaturation. In both cases liposomes were used which were composed of equimolar mixtures of di(18:*n*)PC and di(18:*m*)PE. *m* and *n* represent the number of double bonds of the lipid acyl chains in PE and PC, respectively. *D* was calculated as described under Methods.

take (Fig. 4A) and reduced leucine efflux activity (Fig. 4C) in hybrid membranes composed of *E. coli* PL/egg PC (1:1, w/w). Counterflow uptake was only slightly affected by DEPC. At low concentrations of DEPC, the level of counterflow was even elevated (Fig. 4B). DEPC had no effect on the magnitude of the imposed $\Delta\tilde{\mu}_{H^+}$ (not shown). These results suggest that DEPC mainly acts on H^+-linked leucine transport.

The first-order rate constants (D) for leucine efflux for liposomes (Fig. 5A) and DEPC-treated hybrid membranes (Fig. 5B) were determined for the various di(18:*m*)PE/di(18:*n*)PC mixtures. In DEPC-treated hybrid membranes in which facilitated diffusion is blocked, leucine efflux may take place via the membrane or through the carrier/lipid interface. In liposomes efflux occurs exclusively via the membrane lipids. Both with the liposomes and hybrid membranes, a dramatic increase in passive leucine permeability (decrease of the first-order rate constant, D) was noted with the number of double bonds in the acyl chains of either the PE or PC species.

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 [7–11] containing fatty acid acyl chains with different numbers of unsaturated bonds. The freeze/thaw-sonication technique used to enrich the membranes with exogenous lipids exhibited little specificity for the unsaturation level (Table I) [9,26]. This allowed a convenient and controlled manner of lipid enrichment under conditions that the number of transport systems in the bilayer was kept constant.

The *Bca* carrier exhibits a high activity when reconstituted in mixtures of PC and PE [9]. 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 allows 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*. Lipids with an acyl chain number of 18 provide an optimal thickness of the membrane for the leucine transport system [10]. Therefore, liposomes were used composed of an equimolar mixture of di(18:*m*)PE/di(18:*n*)PC in which *m* and *n* represent the number of double bonds in the acyl chains of the PE and PC lipid species, respectively. Both with counterflow and $\Delta\tilde{\mu}_{H^+}$-driven leucine uptake a decrease in activity was noted with increasing number of double bonds. In this respect the transport activity reduced stronger with increasing degree of acyl chain unsaturation for the PE species than for the PC species. A decrease in transport rate of the Na^+/K^+-ATPase with increasing number of double bonds was noted by Marcus et al. (1986). This effect of unsaturation on enzyme activity was attributed to a reduction of membrane thickness with increasing number of double bonds or a decrease in membrane rigidity causing a destabilization of functional protein conformations [27].

In order to explain the observed effects of acyl chain unsaturation on leucine transport, several possibilities have to be considered.

Membrane fluidity

The membrane fluidity of bacteria is realized by modulating the acyl chain carbon number or the degree of unsaturation of membrane lipids [12,13]. At 25°C, the steady-state TMA-DPH polarization of each of the di(18:*m*)PE/di(18:*n*)PC (m, $n \geq 1$) mixtures used in this study was nearly similar (Table I). In contrast, cholesterol had a strong effect on the mobility of (TMA-)DPH, and this effect was correlated to its impact on the leucine transport activity [11]. An increase in fatty acid acyl chain number also results in a decrease in the mobility of (TMA-)DPH [10]. Under the experimental conditions employed no direct relationship was observed between the degree of unsaturation and the fluidity of a lipid bilayer. Changes brought about in the unsaturation of animal cell membrane phospholipids also had little effect on the steady-state fluorescence polarization of DPH [28]. The steady-state polarization technique does not discriminate between changes in lipid dynamics and lipid order, and only subtle differences have been observed in the dynamic behavior of lipid probes in phospholipid bilayers of varying degrees of unsaturation [23,28–30]. Recent differential scanning calorimetry (DSC) and nuclear magnetic resonance (NMR) studies demonstrate that phospholipid bilayers composed of highly unsaturated lipids display a phase transition temperature that is similar to, or even higher than, that found for their less unsaturated counterparts [31,32]. However, membrane probes are only sensitive to motions that occur over a limited range of frequencies, and in the case of steady-state fluorescence measurements, probes are rigid structures that may not be sensitive to all types of lipid motion [33].

Membrane thickness

Leucine transport activity shows a strong dependency on the acyl chain carbon number [10]. This effect was attributed to changes in membrane thickness and a requirement for an optimal match between the hydrophobic thickness of the membrane and the transport protein [34] as has been suggested for other membrane proteins [35,36]. Membrane thickness is not only affected by the acyl chain carbon number, but the unsaturation level of the acyl chains and the order of

the lipids in the membrane may contribute as well [35,37–40]. Low-angle X-ray diffraction studies of liposomes composed of *cis* mono-unsaturated PC's 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. The addition of one double bond has only a minor effect on the thickness, i.e. a reduction of only 0.1 nm when di(18:1)PC (4.3 nm) and di(18:2)PC (4.2 nm) are compared [37]. The effect of acyl chain unsaturation on leucine transport activity observed in this study is as dramatic as that observed in a previous study in which the acyl chain carbon number was varied [10]. Differences in membrane thickness among the various mixtures of unsaturated lipids may contribute to their effects on transport activity. However, it seems unlikely that the effect of the degree of unsaturation on the transport rates can be solely explained by an effect on membrane thickness.

Lipid affinity

The association of mitochondrial membrane lipids with cytochrome-*c* oxidase and the adenine nucleotide carrier is influenced by the double bond content of the lipid acyl chains. Gradual reduction of the lipid acyl double bonds by palladium-complex-catalyzed hydrogenation results in a looser association of cardiolipin with membrane proteins. Finally, this results in a replacement of cardiolipin by spin-labelled stearic acid in the solvation shell and a reduction of the enzymatic activity of the proteins studied [41]. An effect of unsaturation on the affinity of lipids for membrane proteins has been suggested before [42]. This possibility cannot be definitely excluded, although the effects of the lipid composition on leucine transport activity observed thusfar all appear to involve bulk effects rather than specific lipid interactions [43].

Membrane permeability

Our data suggest a tentative relation between the passive permeability of the membrane for leucine (Fig. 5) and leucine transport activity (Figs. 1 and 3) in membranes containing a high level of unsaturated lipids. The transient behavior of the imposed ΔpH and $\Delta\psi$ suggests that the ion-permeability of the lipid bilayer also increases with increasing number of double bonds (Fig. 2). An increase of H^+/OH^- ion [44] and water permeability [45] with increasing number of double bonds was recognized before. It has been proposed that changes in the ratio of saturated to unsaturated acyl chains and in the length of the acyl chains greatly affect the fluidity of phospholipid bilayers and, hence, their permeability. This increased permeability was attributed to the bends in the acyl chains at the positions of the double bonds. These tends to form less stable Van der Waals interactions with adjacent lipids, keeping the acyl chain region more fluid. Short chain fatty acids occupy less surface area in order to form stable Van der Waals interactions [45]. Leucine transport and permeability appears to be more sensitive to variations in the degree of unsaturation of the PE than PC lipid species. The tendency of PE to adopt a nonbilayer conformation increases with increasing number of double bonds in the acyl chain. Highly unsaturated PC's may even promote the formation of nonbilayer structures due to a lowered ability to stabilize PE in a bilayer conformation. This may affect membrane permeability and thus indirectly affect the activity of the *Bca* transport system. This increase in permeability cannot be attributed to gross destabilization of bilayer structure as with each of the lipid mixtures closed vesicular structures were obtained. It is concluded that alterations in membrane permeability is a major factor in the observed effects of acyl chain unsaturation on leucine transport activity.

The effect of the composition of the phospholipid headgroup, the composition of the fatty acid acyl chains, both with respect to length and degree of unsaturation, and the cholesterol content on the *Bca* carrier of *L. lactis* has been studied by the membrane fusion technique. In summary, the bilayer features affect leucine transport activity in the following order of importance: lipid headgroup (H^+-bonding) > acyl chain carbon number (thickness) > cholesterol (fluidity) > acyl chain unsaturation (indirect by permeability changes).

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