Reconstitution of the Leucine Transport System of *Lactococcus lactis* into Liposomes Composed of Membrane-Spanning Lipids from *Sulfolobus acidocaldarius*†

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Received May 26, 1992; Revised Manuscript Received August 24, 1992

ABSTRACT: The effect of bipolar tetraether lipids, extracted from the thermophilic archaebacterium *Sulfolobus acidocaldarius*, on the branched-chain amino acid transport system of the mesophilic bacterium *Lactococcus lactis* was investigated. Liposomes were prepared from mixtures of monolayer lipids and the bilayer lipid phosphatidylcholine (PC), analyzed on their miscibility, and fused with membrane vesicles from *L. lactis*. Freeze–fracture electron microscopy demonstrates that the bipolar lipids in the hybrid membranes adopted a monomolecular organization at high *S. acidocaldarius* lipid content. Leucine transport activity (i.e., $\Delta\tilde{\mu}_{H^+}$-driven and counterflow uptake) increased with the content of *S. acidocaldarius* lipids and was optimal at a one-to-one (w/w) ratio of PC to *S. acidocaldarius* lipids. Membrane fluidity decreased with increasing *S. acidocaldarius* lipid content. These data suggest that transport proteins can be functionally reconstituted into membranes composed of membrane-spanning lipids provided that membrane viscosity is restricted.

Archaebacteria are a separate group of microorganisms which can be divided into (1) sulfur-dependent thermophiles, (2) methanogens, and (3) halophiles. A major distinction between archaebacteria and eubacteria is the structure of membrane lipids. Archaebacterial lipids possess a number of unusual structural features. They usually contain phytanyl chains derived from saturated C_{20}, C_{25}, or C_{40} isoprenoid alcohols, joined to glycerol or other polyols by ether linkage instead of condensation of fatty acids and alcohols by ester linkage as in the eubacterial and eukaryotic lipids. Ether lipids contain a 2,3-*sn*-glycerol moiety, while the conventional glycerophosphatides or diacylglycerol have the 1,2-*sn*-glycerol stereochemistry. Although the chemical composition of archaebacterial lipids is completely different from conventional ester lipids, the cytoplasmic membrane performs similar functions [for a review, see De Rosa et al. (1991)].

Recently, we have shown the functional reconstitution of eubacterial transport systems into archaebacterial lipids from *Sulfolobus acidocaldarius* (Elferink et al., 1992). *S. acidocaldarius* is an extreme thermophilic bacterium which can grow at temperatures up to 85 °C and a pH of 2–3 (Langworthy & Pound, 1986). The cytoplasmic membrane contains predominantly (95–98%) bipolar lipids composed of macrocyclic tetraethers with two polar heads linked by two hydrophobic C_{40} phytanyl chains with up to four cyclopentane rings per chain. Two main classes of tetraethers can be discriminated, the glycerol dialkylglycerol tetraethers (GDGTs)[1] and the glycerol dialkylnonitol tetraethers (GDNTs). GDGT contains two glycerol moieties, while in GDNT one of the glycerol moieties is substituted by nonitol, a polyol with nine carbon atoms. Most complex lipids occur as phosphoglycolipids in which the sugar residues and phosphate groups are linked to opposite sides of the tetraether molecules. Polar headgroups are restricted to galactose or glucose, or both, and phospho-myo-inositol moieties. Each tetraether lipid molecule spans the entire archaebacterial membrane, resulting in a monolayer membrane organization (De Rosa et al., 1991).

In a previous paper (Elferink et al., 1992), we reported on the functional reconstitution of membrane proteins in liposomes composed of membrane-spanning lipids of *S. acidocaldarius*. Lipids extracted from *S. acidocaldarius* have been partially purified yielding three different lipid fractions with decreasing polarity. The fraction with intermediate polarity (fraction 2) can be used for the functional reconstitution of cytochrome *c* oxidase from beef heart mitochondria. Fusion of the liposomes with membrane vesicles of the Gram-positive bacterium *Lactococcus lactis* yields hybrid membranes capable of oxidase-dependent, $\Delta\tilde{\mu}_{H^+}$-driven leucine transport. In this system, transport activity is determined by various factors such as the activities of the transport system and cytochrome *c* oxidase. We now present a more detailed study on the effect of *S. acidocaldarius* lipids on the activity of the branched chain amino acid transport protein (Bca carrier) of *L. lactis*. Bca carrier activity was assayed with hybrid membranes composed of membrane vesicles of *L. lactis* fused with liposomes made from mixtures of *S. acidocaldarius* lipids and phosphatidylcholine. Leucine transport activity in these hybrid membranes requires the *S. acidocaldarius* lipids, and it is concluded that these monolayer lipids provide a suitable matrix for the reconstitution of the Bca carrier. The results further suggest that the lower activity of the Bca transport system in membrane-spanning lipids compared to natural phospholipid mixtures is due to the rigidity of the membrane-spanning lipids at 25 °C.

† This study has been made possible by the Stichting voor Biofysica with financial support from the Netherlands Organization for Scientific Research (NWO). MGLE is supported by the Foundation for technical Sciences (STW) with financial aid from NWO.

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[1] Abbreviations: Bca, branched chain amino acid; DMPC, dimyristoyl-PC; DPH 1,6-diphenyl-1,3,5-hexatriene; N-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid); PL, phospholipid; $\Delta\tilde{\mu}_{H^+}$, 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 difference; ΔpH, transmembrane pH difference; GDGT, glycerol dialkylglycerol tetraether lipid; GDNT, glycerol dialkylnonitol tetraethers.

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EXPERIMENTAL PROCEDURES

Bacteria Growth Conditions and Isolation of Membrane Vesicles. *L. lactis* sp. *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 (Otto et al., 1983; Poolman et al., 1987). Membrane vesicles were obtained by osmotic lysis (Otto et al., 1982) and stored in liquid nitrogen for later use.

Materials. Phospholipids were obtained from Sigma Chemical Co. (St. Louis, MO). All lipids were checked for purity with thin-layer chromatography (TLC). N-NBD-PE, 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 Nuclear (Dreieich, FRG). *S. acidocaldarius* (DSM 639) cells were kindly supplied by Dr. G. Schäfer (Medical University of Lübeck, Germany).

Purification of S. acidocaldarius Lipids. Lipids were extracted from freeze-dried *S. acidocaldarius* cells essentially as described (Lo & Chang, 1990). Freeze-dried cells (1.5 g) were soxhlet extracted with 400 mL of chloroform/methanol (1:1 v/v) during 12 h. The crude lipid extract was dried with a rotary evaporator, resuspended in 20 mL of methanol/water (1:1 v/v), and sonicated in a bath sonicator for about 1 h to facilitate dispersion. Homogenization was completed with a probe sonicator (MSE Scientific Instruments, West Sussex) under a N_2 atmosphere. Portions of 5 mL of this suspension were transferred to a Prep Sep C18 column (Waters, Millipore, Milford, MA), and lipids were eluted with 40 mL of methanol/water (1:1 v/v) and 40 mL of chloroform/methanol/water (2:5:2 v/v/v). The second lipid fraction was dried, resuspended in chloroform/methanol/water (65:25:4 v/v/v/), and stored at 4 °C until use.

Liposome Formation and Membrane Fusion. The formation of liposomes (7.5 mg of lipid) and subsequent fusion with *L. lactis* membrane vesicles (0.75 mg of protein) by freeze/thaw–sonication was performed as described (In't Veld et al., 1991).

Transport Assays. $\Delta\tilde{\mu}_{H^+}$-driven L-leucine transport was performed essentially as described (In't Veld et al., 1991). 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 of valinomycin/mg of protein. Membranes were concentrated by centrifugation for 45 min at 53 000 rpm ($210000g_{max}$) in a Beckman type 75 Ti rotor at 5 °C. Samples of 2 μL of the concentrated membrane suspension (8–10 mg of protein/mL) were diluted into 200 μL of solution containing 20 mM sodium phosphate, pH 7.0, 100 mM piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES), and 1.5 μM L-[U-^{14}C]leucine. At different times, the reaction was arrested by dilution into 2 mL of 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 of 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 (8–10 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 quadruplicate. 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 (De Vrij et al., 1986). TPP^+, however, binds to membranes in a concentration-dependent manner. Therefore, a model is applied to account for increased binding when TPP^+ is accumulated by the membrane vesicles (Lolkema et al., 1982). $\Delta\psi$ was calculated according to

$$\Delta\psi = \left[\frac{c_o(c_e - 1)^{-1} + x(1 - 0.5f_{cm}K_{cm})}{x(1 + 0.5f_{cm}K_{cm})}\right] \quad (1)$$

where c_o represents the TPP^+ concentration before binding or uptake has occurred, and c_e represents the external TPP^+ concentration after the generation of a $\Delta\psi$. x and the term $f_{cm}K_{cm}$ are the fractional internal volume of the liposomes and the binding constant of TPP^+ to the membranes, respectively. For $\Delta\psi$ measurements, 10 μL of the concentrated membrane suspension used for imposed $\Delta\tilde{\mu}_{H^+}$-driven uptake was diluted into 1 mL of a buffer containing 20 mM sodium phosphate (pH 7.0), 100 mM sodium acetate, 2 μM TPP^+, and 6 mM $MgSO_4$. The $\Delta\psi$ was dissipated by the addition of 50 nM nigericin. The maximum level of TPP^+ uptake was taken for the calculation of $\Delta\psi$. The TPP^+ binding constant was determined in a similar experiment, except that the buffer contained potassium instead of sodium salts.

Fluorescence Polarization Measurements. Labeling of membranes with DPH (1,6-diphenyl-1,3,5-hexatriene) and TMA-DPH (1-[4-trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene) for steady-state fluorescence polarization (r_{ss} measurements was carried out as described (In't Veld et al., 1991). The fluorescence polarization was measured in a Perkin-Elmer LS-50 spectrofluorimeter with a temperature-controlled cuvette. The temperature in the cuvette was slowly increased to measure temperature-dependent polarization changes. Excitation was at 360 nm. The fluorescence intensity at 430 nm was measured parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the emitted light. Equation 2 was used to calculate r_{ss} (Shinitzky & Inbar, 1976, 1978) from the corrected fluorescence intensities.

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

Differential Scanning Calorimetry. For differential scanning calorimetry (DSC), 8 mg of lipids were hydrated in 100 mM NaCl, supplemented with 25 mM piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid) (PIPES), pH 7.0, and concentrated by centrifugation for 45 min at 53 000 rpm ($121000g_{max}$) in a Beckman type 75 Ti rotor at 18 °C. The pellet was transferred to an aluminum sample pan which was subsequently sealed in a press. Data were taken on a Perkin-Elmer DSC-7 scanning calorimeter at a scanning rate of 10 °C/min.

Sucrose Gradient Centrifugation. Discontinuous sucrose gradients were performed in Beckman ultracentrifugation tubes on top of a 65% (w/v) sucrose cushion. The discontinuous gradients were prepared from the following sucrose concentrations made in 10 mM Tricine (pH 8.0) containing 100 mM KCl and 1 mM EDTA: 45% (1 mL), 35% (1 mL), 28% (1 mL), 23% (1 mL), 18% (1 mL), 13% (1 mL), 8% (1 mL), and 3% (1 mL). Hybrid membranes or a mixture of liposomes and membrane vesicles (6 mg of lipid and 0.6 mg of protein) were layered on the top of the gradients and centrifuged for 19 h at $145000g_{max}$ in an SW 41 rotor at 18 °C. Membrane vesicles and liposomes were fluorescently

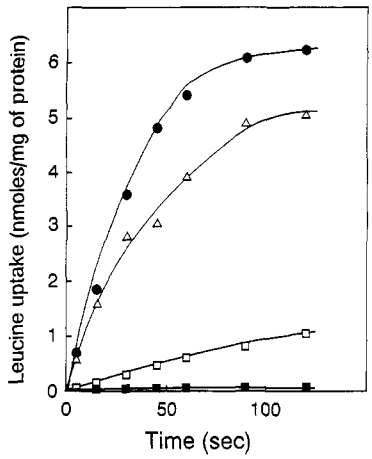

FIGURE 1: $\Delta\tilde{\mu}_{H^+}$-Driven leucine transport in hybrid membranes obtained by fusion of *L. lactis* membrane vesicles with liposomes composed of *S. acidocaldarius* lipids (□), *S. acidocaldarius* lipids/*E. coli* PL (1:1 w/w) (●), and *S. acidocaldarius* lipids/egg PC (1:1 w/w) (△); control showing leucine uptake by hybrid membranes composed of *S. acidocaldarius* lipids (■) when no $\Delta\mu_{H^+}$ is imposed. Similar uptake levels were observed with mixtures of *S. acidocaldarius* lipid with egg PC or *E. coli* PL. Details are as described under Experimental Procedures.

labeled with octadecyl rhodamine B chloride (R_{18}) and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (N-NBD-PE), respectively. *L. lactis* membrane vesicles were labeled with R_{18} essentially as described before (Hoekstra et al., 1984). A solution of 50 nmol of R_{18} in 5 μL of EtOH was added under extensive vortexing to a 1-mL suspension of *L. lactis* membrane vesicles (5 mg of protein). The mixture was incubated in the dark for 1 h at room temperature. Nonincorporated R_{18} was removed by washing three times with 50 mM potassium phosphate, pH 7.0. N-NBD-PE was incorporated into liposomes at concentration of 6 μg/mg of lipid during the preparation of the liposomes. The distribution of vesicle protein and liposomal lipid along the gradient was determined by assaying the fluorescence of these labels in the presence of 1% (v/v) Triton X-100. Excitation and emission wavelengths were 560 and 590 nm (R_{18}) (Hoekstra et al., 1984) and 475 and 530 nm (NBD-PE) (Struck et al., 1981), respectively. Sucrose densities were estimated from the refractive indices.

Other Analytical Procedures. Protein was determined by the method of Lowry et al. (1951) in the presence of 0.5% (w/v) sodium dodecyl sulfate (Dulley & Grieve, 1975). Bovine serum albumin was used as a standard.

RESULTS

$\Delta\tilde{\mu}_{H^+}$-Driven Leucine Uptake in Fused Membranes Containing S. acidocaldarius Monolayer Lipids. Liposomes were prepared from a lipid fraction with intermediate polarity extracted from *S. acidocaldarius* freeze-dried cells (Elferink et al., 1992) and fused with *L. lactis* membrane vesicles. The uptake of leucine was assayed upon imposition of a $\Delta\tilde{\mu}_{H^+}$ by the use of an outwardly directed potassium acetate concentration gradient in the presence of the ionophore valinomycin. This method establishes both a membrane potential ($\Delta\psi$) and a pH gradient (ΔpH) across the membrane. Hybrid membranes composed of the membrane-spanning lipids exhibited a relatively low leucine transport activity (Figure 1, □), which was, however, significantly higher than the level of leucine uptake in the absence of an imposed $\Delta\tilde{\mu}_{H^+}$ (■). However, high transport activities were found when the *S. acidocaldarius* lipids were mixed (1:1, w/w) with *Escherichia coli* phospholipids (●) or egg phosphatidylcholine (PC) (△). Previous results have shown that the lipid headgroup of phosphatidylethanolamine (PE), a constituent of *E. coli* PL, is able to activate the Bca carrier, while PC is not (Driessen et al., 1988) (see also Figure 5). It is, therefore, concluded that the lipid mixture from *S. acidocaldarius* contains lipid species which are able to support the activity of the Bca carrier.

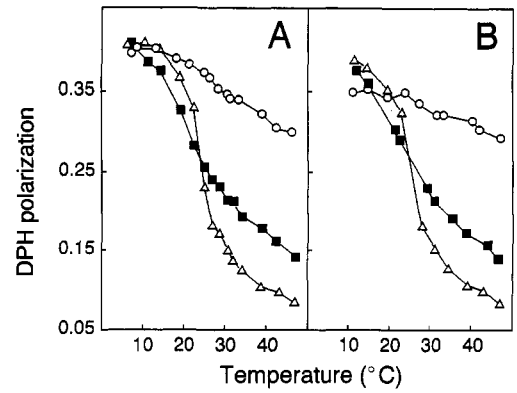

FIGURE 2: Heating (A) and cooling (B) temperature scan of the steady-state DPH anisotropy (r_{ss}). Liposomes are composed of DMPC (△), *S. acidocaldarius* lipids (○), and DMPC/*S. acidocaldarius* lipids (1:1 w/w) (■).

Monolayer and Bilayer Lipids Are Miscible. By mixing PC and *S. acidocaldarius* lipids, membranes are formed from an unusual blend of bilayer and monolayer lipids. In order to obtain stable liposomal structures, complete miscibility of the two different lipid species is required. Miscibility of these lipids was studied by measuring the gel to liquid-crystalline phase transition temperature by the use of 1,6-diphenyl-1,3,5-hexatriene (DPH) steady-state polarization. This transition is characterized by an abrupt change in fluidity with increasing temperature (Shinitzky & Inbar, 1978). The thermotropic phase transition behavior of DPH in liposomes composed of synthetic dimyristoyl-PC (DMPC) shows a sharp transition at 25 °C (Lentz et al., 1976), both in the heating (Figure 2A) and cooling (Figure 2B) temperature profiles (△). The same temperature profile for *S. acidocaldarius* lipids exhibits no sharp transition between 5 and 50 °C (○), as expected for a natural lipid mixture containing different lipid species. However, when DMPC and *S. acidocaldarius* lipids are mixed in equal amounts (1:1 w/w), the temperature range of the transition of DMPC appears to be broadened (■). Suppression of the DMPC transition by *S. acidocaldarius* lipids is indicative for mixing of both lipid species.

A disadvantage of extrinsic membrane probes such as DPH is that their response may differ depending on the lipid species used. In this respect, *S. acidocaldarius* lipids markedly quenched the fluorescence of DPH (not shown). An independent technique was used to establish mixing of these lipids. Differential scanning calorimetry (DSC) monitors changes in the physical state of lipids, such as the transition from the gel to liquid-crystalline phase. This transition occurs at a characteristic temperature and enthalpy change. The heating and cooling temperature scans for DMPC liposomes show a sharp peak at 29 and 24.5 °C, respectively (Figure 3, lines a and b). *S. acidocaldarius* lipids show no transition in this narrow temperature range, i.e., between 20 and 80 °C (line c). However, the DSC profiles of the mixture of both lipid species show not only a broadening of the transition peak but also a down shift in transition temperature by 2–3 °C. These data demonstrate that the bipolar lipids of *S. acidocaldarius* and the bilayer lipids (DM)PC are miscible.

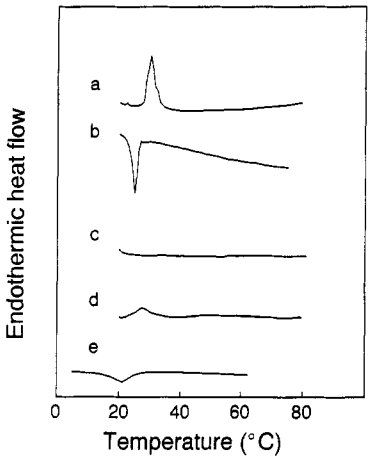

FIGURE 3: Differential scanning calorimetry profiles of liposomes composed of DMPC (A, heating scan; B, cooling scan), *S. acidocaldarius* lipids (C, heating and cooling scan), and DMPC/*S. acidocaldarius* lipids (1:1 w/w) (D, heating scan; E, cooling scan).

Fusion of Bacterial Membrane Vesicles with Monolayer Liposomes. Liposomes composed of *S. acidocaldarius* bipolar lipids and egg PC were fused with *L. lactis* membrane vesicles and characterized by means of sucrose gradient centrifugation. Mixtures of liposomes and membrane vesicles (i.e., nonfused) and freeze/thaw–sonication-treated membranes (i.e., fused) were applied to the gradients. To visualize the positions of the membrane vesicles of *L. lactis* and liposomes in the gradients, membranes were labeled with the fluorescent probes R_{18} and N-NBD-PE, respectively. Fusion between the membrane vesicles and *S. acidocaldarius* liposomes is evident from the repositioning of the membrane vesicle (red) and liposomal (yellow) band to a single membrane band with intermediate density (compare Figure 4, panels E and F) and blended color (orange) (not shown). A similar conclusion can be drawn with respect to the fusion between *L.* lactis membrane vesicles and liposomes composed of *E. coli* PL/egg PC (1:1 w/w) (A, B) or *S. acidocaldarius* lipids/egg PC (1:1 w/w) (C, D). It is of interest to note that liposomes containing *S. acidocaldarius* lipids exhibit a higher density than *E. coli* PL/egg PC (1:1 w/w) liposomes. These data indicate that the monolayer *S. acidocaldarius* liposomes fuse with *L. lactis* membrane vesicles when a mechanical fusion-inducing method of freeze/thaw–soniciation is used.

S. acidocaldarius Monolayer Lipids Support $\Delta\tilde{\mu}_{H^+}$-Driven and Counterflow Leucine Uptake in Fused Membranes. Liposomes were made from mixtures of *S. acidocaldarius* lipids and egg PC, fused with *L. lactis* membrane vesicles, and assayed for $\Delta\tilde{\mu}_{H^+}$-driven leucine transport activity (Figure 5A). As a control, hybrid membranes were prepared composed of *E. coli* PL/egg PC (1:1 w/w) (▲). With PC alone only a low level of leucine uptake was observed (○). Uptake increased when the fraction of *S. acidocaldarius* lipids was increased and appeared to be maximal at 50%. A further increase of the monolayer lipid concentration, i.e., up to 100%, resulted in a decrease in the uptake levels (Figure 5B). Similar results were obtained when the initial rate of uptake was determined from samples taken during the first 10 s (not shown). These results provide further evidence that the bipolar lipids of *S. acidocaldarius* activate the Bca carrier.

Since the transport activity is a direct function of the magnitude of the imposed $\Delta\tilde{\mu}_{H^+}$, the ability of the hybrid membrane to sustain an $\Delta\tilde{\mu}_{H^+}$ was tested by direct measurements of the $\Delta\psi$. $\Delta\psi$ was assessed with an ion-selective electrode which monitors the external concentration of the lipophilic cation tetraphenylphosphonium (TPP^+) (Table I).

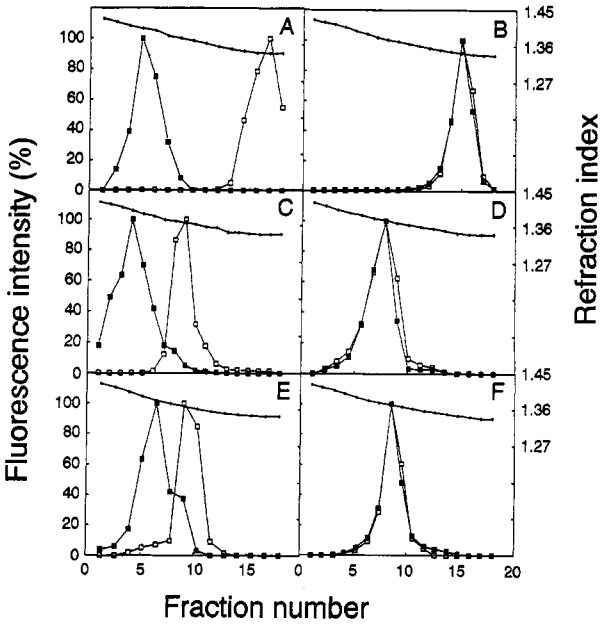

FIGURE 4: Sucrose gradient centrifugation of *L. lactis* membrane vesicles mixed with liposomes composed of *E. coli* PL/egg PC (1:1 w/w) (A), *S. acidocaldarius* lipids/egg PC (1:1 w/w) (C), or *S. acidocaldarius* lipids (E) and fused by freeze/thaw–sonication (B, D. and F, respectively). After centrifugation, gradients were fractionated and assayed for the positions of the membrane vesicles (■), liposomes (□), and refractive indices (●). Membrane vesicles were detected by the fluorescence [in arbitrary units (%)] of the marker R_{18}, which was previously reacted with the membrane vesicles. Liposomes were detected by NBD-PE fluorescence [in arbitrary units (%)], which was incorporated at a concentration of 6 μg/mg of lipid during the preparation of the liposomes.

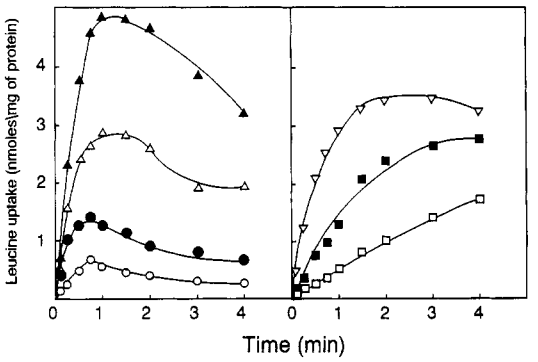

FIGURE 5: $\Delta\tilde{\mu}_{H^+}$-Driven leucine transport in hybrid membranes obtained by fusion of *L. lactis* membrane vesicles with liposomes composed of *E. coli* PL/egg PC (1:1 w/w) (▲) and *S. acidocaldarius* lipids/egg PC in weight ratios of 0:1 (○), 1:3 (●), 1:1 (△), 3:1 (▽), 9:1 (■), and 1:0 (□).

At all lipid compositions, a high transient $\Delta\psi$ was generated which collapsed upon the addition of nigericin (not shown). $\Delta\psi$ was calculated using a correction for concentration-dependent binding of TPP^+ to the membranes assuming symmetric binding of TPP^+ to the membranes (Lolkema et al., 1982; De Vrij et al., 1986). Nonspecific binding of TPP^+ increases dramatically with increasing *S. acidocaldarius* lipid content of the hybrid membranes (Table I). However, the $\Delta\psi$ calculated remained essentially independent of the lipid composition of the hybrid membranes, although lower $\Delta\psi$ values were observed with hybrid membranes containing *S. acidocaldarius* lipids only. These results show that hybrid membranes containing a substantial amount of bipolar lipids are capable of sustaining an imposed $\Delta\psi$.

The effect of *S. acidocaldarius* lipids on leucine transport activity was also studied with the counterflow assay. Counterflow uptake is independent on the ion-permeability of the

Table I: $\Delta\psi$ Values in Hybrid Membranes upon the Imposition an Valinomycin-Mediated Outwardly Directed Potassium Diffusion Gradient

composition of liposomes fused with *L. lactis*[a]	$\Delta\psi$ (mV)[b]	bindings constant[c]
E. coli PL/egg PC (1:1)	−82	50
egg PC	−89	16
egg PC/*S. acidocaldarius* (3:1)	−118	18
egg PC/*S. acidocaldarius* (1:1)	−89	70
egg PC/*S. acidocaldarius* (1:3)	−82	126
egg PC/*S. acidocaldarius* (1:9)	−104	170
S. acidocaldarius	−72	198

[a] Lipid composition in weight ratios. [b] The $\Delta\psi$ was calculated from the maximum level of TPP^+ uptake and corrected for concentration-dependent binding of TPP^+ to the membranes. [c] Symmetric binding of TPP^+ is assumed.

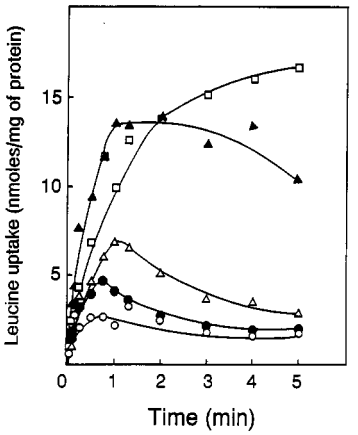

FIGURE 6: Leucine counterflow uptake in hybrid membranes obtained by fusion of *L. lactis* membrane vesicles with liposomes composed of *S. acidocaldarius* lipids/egg PC in weight ratios of 0:1 (○), 1:3 (●), 1:1 (△), 3:1 (▲), and 1:0 (□).

membrane. At saturating concentrations of leucine at both sides of the membrane, the initial rate of counterflow uptake reflects the exchange activity of the transport system (Driessen et al., 1987a). Counterflow uptake was performed with 5 mM and 50 μM leucine at the inside and outside, respectively. [^{14}C]Leucine was transiently accumulated by the hybrid membranes (Figure 6). Hybrid membranes which contained 50% or more *S. acidocaldarius* lipids showed a high counterflow activity. The initial rate of counterflow uptake, i.e., samples taken after 5 s, increased with increasing amounts of *S. acidocaldarius* lipid (not shown). Maximal activity was observed with hybrid membranes composed of egg PC and *S. acidocaldarius* lipid mixed in a 1-to-3 ratio (w/w). These results provide direct evidence for the activating effect of membrane-spanning lipids on the activity of the Bca carrier and demonstrate that the Bca carrier is active when reconstituted into *S. acidocaldarius* lipids.

S. acidocaldarius Lipids Decrease the Membrane Fluidity of Hybrid Membranes. The steady-state fluorescence polarization (r_{ss}) of DPH and 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) at 25 °C was measured for hybrid membranes composed of various mixtures of *S. acidocaldarius* lipids and egg PC. Fluorescence polarization increased with increasing amounts of monolayer lipids of *S. acidocaldarius* in the hybrid membranes (Figure 7), suggesting an increase in membrane viscosity with the amount of monolayer lipid.

DISCUSSION

The transport system for branched chain amino acids (Bca carrier) of *L. lactis* catalyzes the uptake of L-leucine,

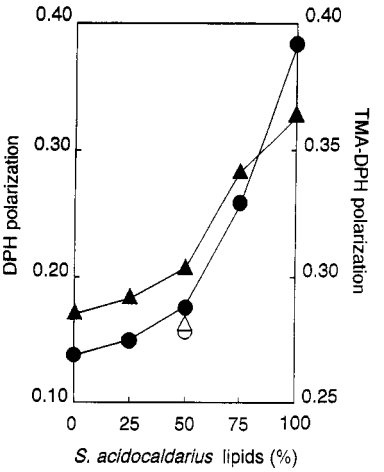

FIGURE 7: DPH (●) and TMA-DPH (▲) anisotropy (r_{ss}) in hybrid membranes obtained by fusion of *L. lactis* membrane vesicles with liposomes composed of different ratios of *S. acidocaldarius* lipids/egg PC. DPH (○) and TMA-DPH (△) r_{ss} values for fused membranes composed of egg PC/*E. coli* PL (1:1 w/w) are indicated for comparison.

L-isoleucine, and L-valine in symport with one proton (Driessen et al., 1987a,b; Konings et al., 1989). The membrane of *L. lactis* consists of about 50% phospholipids (phosphatidylglycerol and cardiolipin) and 50% (phospho-)glycolipids (Driessen et al., 1988). The most important fatty acids present are palmitic acid ($C_{16:0}$), oleic acid ($C_{18:1}$), and the cyclopropane ring containing lactobacillic acid ($C_{19\Delta}$) (In't Veld et al., 1991). Lipid–protein interactions of the Bca carrier have been studied with model systems in which isolated membrane vesicles were enriched with exogenous lipids upon fusion with liposomes. These studies demonstrated a lipid requirement which could be attributed to bulk composition of the membrane with aminophospholipids and glycolipids as activating species. The transport activity of the Bca carrier depends not only on the composition of the phospholipid headgroups (Driessen et al., 1988) but also on the fatty acid acyl chains (In't Veld et al., 1991, 1992) and on the "fluidity" of the membrane (Zheng et al., 1988). Maximal transport activity was observed with phospholipids containing fatty acid acyl chains of 18 C atoms.

We recently reported on the functional reconstitution of cytochrome *c* oxidase from beef heart mitochondria (Elferink et al., 1992) and bacteriorhodopsin (Elferink et al., unpublished results) into lipids extracted from the archaebacterium *S. acidocaldarius*. Upon fusion with *L. lactis* membrane vesicles, leucine was accumulated by the $\Delta\tilde{\mu}_{H^+}$, generated by cytochrome *c* oxidase activity. However, leucine uptake activity observed with hybrid membranes composed of solely *S. acidocaldarius* lipids was much lower compared to the natural *E. coli* phospholipid mixture. Since transport activity in this system depends on various factors such as the cytochrome *c* oxidase activity, ion permeability, and phospholipid composition of the membrane, it was not possible to determine to what extend the *S. acidocaldarius* lipids support functioning of the leucine transport system. Using the same fusion approach, we now demonstrate that the *S. acidocaldarius* lipids support leucine transport activity. Membrane vesicles of *L. lactis* were fused with liposomes containing different mixtures of the monolayer lipids and the bilayer lipid phosphatidylcholine. The steady-state DPH polarization (Figure 2) and differential scanning calorimetry (Figure 3) measurements suggest that both lipid species are miscible. Sucrose gradient density centrifugation (Figure 4) indicates that the freeze–thaw/sonication method yields hybrid mem-

branes. Membranes composed of egg PC are not able to activate the Bca carrier as shown by the low counterflow and $\Delta\tilde{\mu}_{H^+}$-driven uptake of leucine (Driessen et al., 1988) (Figures 6 and 7). Uptake activity increased with the *S. acidocaldarius* lipid content of the hybrid membranes, although inhibition was observed at higher concentrations. Membranes containing a high level of *S. acidocaldarius* lipid retained the ability to maintain an imposed $\Delta\psi$, although the calculated $\Delta\psi$ value was reduced compared to the other lipid mixtures. We noted that the permeability of the membranes toward the $\Delta\psi$ probe TPP^+ decreased with increasing amounts of *S. acidocaldarius* lipids, most dramatically when pure *S. acidocaldarius* lipids were used. This decreased TPP^+ permeability may result in an underestimation of the true $\Delta\psi$, as conditions may prevail in which equilibrium is not yet reached while $\Delta\psi$ already starts to decrease. We assume, however, that initially the imposed $\Delta\psi$ (and $\Delta\tilde{\mu}_{H^+}$) is equal for each lipid mixture. In this respect, counterflow uptake is considered to be a more reliable measure for activity as it does not depend on $\Delta\tilde{\mu}_{H^+}$ (Figure 6). Though inhibition of counterflow activity was less pronounced at a high monolayer lipid concentration compared to $\Delta\tilde{\mu}_{H^+}$-driven uptake, the highest activity was observed when a mixture of egg PC and *S. acidocaldarius* lipid was used. This inhibition of transport activity may relate to the increased rigidity of the membrane at high monolayer lipid concentrations (Figure 7). In this respect, counterflow activity is less dependent on membrane fluidity (Zheng et al., 1988), which may explain the high counterflow activity observed when the Bca carrier is reconstituted in membrane-spanning lipids only. The rigidity of monolayer lipids has been attributed to the formation of a monolayer by tetraethers which span the membrane. The two sides of the membrane are covalently bound, thereby preventing the hydrophobic core from disruption (Russell & Fukunaga, 1990). This monolayer organization is also evident from electron micrographs of hybrid membranes composed of *S. acidocaldarius* lipid/egg PC (9:1 w/w) (In't Veld et al., unpublished data). A similar freeze–fracturing behavior is observed with liposomes composed of mixtures of *S. acidocaldarius* and *E. coli* lipid when the amount of bilayer lipid does not exceed 50% (w/w) (M. G. L. Elferink, unpublished data). Another genotypic thermal adaptation of the thermophilic archaebacterial lipids is the number of cyclopentane rings per phytanyl chain which increases with the thermal optimum for growth. Cyclization shortens the phytanyl chains and increases their rigidity (Russell & Fukunaga, 1990). The inhibiting effect of high concentrations of monolayer lipids may be very similar to that observed for cholesterol (Zheng at al., 1988). Nevertheless, despite this inhibition, a significant leucine transport activity remains when hybrid membranes are composed solely of monolayer lipids.

At lower concentrations of the membrane-spanning lipids ($\leq$50% w/w), the impact on membrane fluidity is only limited, and activity is mainly determined by the activating effect of the lipid headgroups introduced with the monolayer lipids. The polar headgroup of tetraether lipids exists mainly of hydrogen-bond-forming sugar residues, i.e., galactose or glucose, or both, and phospho-myo-inositol moieties (De Rosa et al., 1991). In previous studies, it was concluded that the formation of hydrogen bonds between the carrier and the surrounding lipids are a prerequisite for a functional transport system (Driessen et al., 1988). Aminophospholipids and glycolipids are activators, whereas PC is not able to activate the leucine transport protein. The membrane of thermophilic archaebacteria contains a high proportion of glycosylated lipids. There is a general increase in glycolipid content, and its level increases with increasing growth temperature. It has been suggested that this thermal dependency of the content of sugar-containing lipids increases the hydrogen-bonding capacity of the membrane surface, thereby stabilizing the membrane at high temperatures (Russell & Fukunaga, 1990). Additional interactions with cell wall components may further stabilize the cell envelope. Analogously, this increased capacity to form hydrogen bonds may be important in the stabilization of the integral membrane proteins which function in these monolayers.

In this paper, we demonstrate that the natural lipid mixture of the extreme thermophilic archaebacterium *S. acidocaldarius*, which is organized as a monolayer, functions as a suitable matrix for the reconstitution of the Bca carrier from a mesophilic bacterium, in this case *L. lactis*. Optimal activity can be obtained by mixing monolayer with bilayer lipids to obtain membrane structures that can be used to study transport activities at lower temperatures. The functional reconstitution of membrane proteins into liposomes composed of monolayer lipids provides a new tool in the study of the mechanisms of solute and macromolecule translocation across biological membranes.

ACKNOWLEDGMENT

We thank Dr. G. Schäfer for supplying us with *S. acidocaldarius* cells. We also thank G. Alberda van Ekenstein for the help with the DSC experiments.

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Registry No. Leucine, 61-90-5.