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Acidic phospholipids are required during solubilization of amino acid transport systems of *Lactococcus lactis*

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The branched-chain amino acid transport system of *Lactococcus lactis* was solubilized with *n*-octyl β-D-glucopyranoside and reconstituted into proteoliposomes. Transport activity was recovered only when solubilization was performed in the presence of acidic phospholipids. Omission of acidic phospholipids during solubilization resulted in an inactive transport protein and the activity could not be restored in the reconstitution step. Similar results have been obtained for the arginine/ornithine exchange protein from *Pseudomonas aeruginosa* and *L. lactis*. Functional reconstitution of the transport protein requires the presence of aminophospholipids or glycolipids in the liposomes (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). We propose that during the detergent solubilization the acidic phospholipids protect the transport systems against denaturation by preventing delipidation.

Introduction

Membrane transport proteins catalyze the specific transfer of solutes across the membrane. These proteins are embedded in the cytoplasmic membrane and their activities are modulated by the surrounding lipid bilayer. Protein–lipid interactions of membrane transport proteins is most conveniently studied *in vitro* using proteoliposomes of defined lipid composition. The lipid environment can be modulated by different means. Firstly, membrane vesicles can be fused with liposomes using a simple freeze/thaw-sonication method [1–3]. Secondly, membrane proteins can be extracted from the cytoplasmic membrane with detergents either in the absence or presence of exogenous phospholipids and subsequently reconstituted into liposomes of defined lipid composition. An important difference between the two methods is the use of detergent. *n*-Octyl β-D-glucopyranoside is widely used for the solubilization of bacterial transport proteins. It can be easily removed because of its high critical micellar concentration [4,5]. In many cases, recovery of activity is significantly improved when osmolytes [6,7] and phospholipids [8–10] are present in the solubilization step. It has been suggested that these compounds protect membrane proteins against denaturation during solubilization [6–8] thereby preventing aggregation and precipitation of transport proteins [11]. The solubilization/reconstitution approach has been used to study lipid–protein interaction for a number of different bacterial transport proteins, such as the lactose carrier of *Escherichia coli* [12,13] and the Na^+-dependent leucine transport system of *Pseudomonas aeruginosa* [14,15].

In previous studies, we applied the method of membrane fusion to investigate systematically the role of the phospholipid polar headgroup [16] and fatty acid acyl chain [17] composition on the activity of the branched chain amino acid transport system (Bca carrier) of *Lactococcus lactis*. These studies revealed a requirement for aminophospholipids (phosphatidylethanolamine and phosphatidylserine) or glycolipids, suggesting that formation of hydrogen bonds between membrane lipids and the transport protein is essential for the stabilization of the carrier in an active confor-

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Abbreviations: Bca, branched-chain amino acid; Δp, protonmotive force; CL, cardiolipin; DGDG, digalactosyldiglyceride; DOPC, dioleoylphosphatidylcholine; DOPA, dioleoylphosphatidic acid; DOPE, dioleoylphosphatidylethanolamine; DOPG dioleoylphosphatidylglycerol; DOPS, dioleoylphosphatidylserine; EcoPL, *E. coli* phospholipid; MGDG, monogalactosyldiglyceride; octyl glucoside, *n*-octyl β-D-glucopyranoside; PI, phosphatidylinositol.

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mation [16]. Another parameter essential for the stabilization of the Bca carrier is the degree of matching of the hydrophobic thickness of the lipid bilayer and the protein [17].

In this study the lipid–protein interaction of the Bca carrier has been further analyzed using the solubilization/reconstitution method. The phospholipid required for the solubilization of a functional Bca carrier from its native membrane differs from the lipids required for functional reconstitution. While aminophospholipids or glycolipids have to be present in the final proteoliposomes, acidic phospholipids are required during the solubilization step. The results indicate that acidic phospholipids are needed for maintaining an active conformation of transport proteins outside their natural environment.

Methods

Bacteria, growth conditions and isolation of membrane vesicles

Lactococcus lactis subsp. *lactis* ML_3 was grown on a chemically defined medium with 1% (wt/vol) galactose and 25 mM L-arginine at 30°C and pH pH 6.4 [19,20]. Membrane vesicles were obtained by osmotic lysis [21] and stored in liquid nitrogen for later use. Isolation of membrane vesicles of *Escherichia coli* JC182-5 [22] containing plasmid pME 3719 [23] carrying the *arcD* gene coding for an arginine/ornithine exchange transport system of *Pseudomonas aeruginosa* [38] was performed as described [24].

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 [17].

Solubilization and reconstitution

Membrane vesicles (1.5 mg of protein) were solubilized with 1.25% (w/v) *n*-octyl β-glucopyranoside [8] in the presence of 9.75 μmol phospholipid for *L. lactis* membrane vesicles or 29.3 μmol for *E. coli* membrane vesicles (in 4% (w/v) octyl glucoside), 20% (v/v) glycerol [6] in a final volume of 1.5 ml 50 mM potassium phosphate (pH 7.0). The suspension was incubated for 30 min on ice and centrifuged for 1 h at 43000 rpm ($225000 \times g_{max}$) in a Beckman type SW 50.1 rotor at 5°C. A suspension of 9.75 μmol phospholipids in 4% (w/v) octyl glucoside was added to the cleared supernatant (1.5 ml). The final octyl glucoside concentration was adjusted to 1.5% (w/v) with 50 mM potassium phosphate (pH 7.0) to obtain a transparent solution of mixed micelles. The suspension was kept on ice for 10 min. Detergent was removed either by dialysis (when natural phospholipids were used for solubilization and reconstitution) or dilution (when synthetic lipids were used for solubilization and/or reconstitution). Dialysis was performed against a 1000-fold volume of 50 mM potassium phosphate (pH 7.0), at 4°C for 24 h with three changes. Reconstitution by dilution was performed by rapidly injecting the membrane protein suspension into 50 mM potassium phosphate (pH 7.0), such that a 35-fold dilution was obtained. To assay leucine counterflow, 5 mM leucine was included in the dilution buffer. The dilution buffer contained 0.5 mM ornithine for the assay of arginine/ornithine exchange. The suspension was carefully stirred for 5 min and proteoliposomes were collected by centrifugation for 2.5 h at 27000 rpm ($90000 \times g_{max}$) in a Beckman type 35 Ti rotor. Proteoliposomes were 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

Δp-driven L-leucine transport and L-leucine counterflow were performed as described [17]. The concentrated membrane suspensions contained approx. 8.5 mg of protein/ml for fused membranes and 4.5 mg of protein/ml for proteoliposomes.

For the assay of arginine/ornithine exchange, proteoliposomes were loaded with 0.5 mM ornithine (see dilution method) and concentrated. Samples of 2 μl (approx. 4.6 mg protein/ml for *L. lactis* antiporter and 3.1 mg protein/ml for *P. aeruginosa* exchanger) were diluted into 100 μl 50 mM potassium phosphate (pH 7.0), containing 2.9 μM L-[U-^{14}C]arginine. Transport assays were performed at 5°C to allow an accurate assessment of the initial transport rates after 10 s.

Other analytical procedures

The trapped volume of fused membranes and proteoliposomes was determined with the fluorophore calcein [17]. Protein in fused membranes was determined by the method of Lowry et al. [25] in the presence of 0.5% (v/v) sodium dodecylsulfate [26]. The Pierce micro BCA protein assay [27] was used for proteoliposomes. Bovine serum albumin was used as a standard. Lipids were analyzed by two-dimensional thin-layer chromatography on precoated silica gel plates (Merck, Kieselgel 60) using the following solvent system: A, $CHCl_3$/MeOH/ammonia/water (90:54:5.5:5.5, v/v) and B, $CHCl_3$/MeOH/acetic acid/water (90:40:12:2, v/v). Chromatograms were stained with I_2 vapour and analyzed for phospholipid phosphorous [28].

Materials

Synthetic phospholipids were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL, USA). All lipids

were checked for purity with thin-layer chromatography (TLC). Crude *E. coli* phospholipids (PL) were obtained from Sigma Chem. Co. (St. Louis, MO, USA) and acetone/ether-washed by a modification of the method of Kagawa and Racker [18]. *E. coli* Cl, MGDG and DGDG were obtained from Sigma. L-[U-^{14}C] Leucine (12.4 TBq/mol) and L-[U-^{14}C]arginine (11 TBq/mol) were purchased from New England Nuclear (Dreieich, FRG). Bovine brain PS and bovine liver PI was a gift of Dr. J. Wilschut (Department of Physiological Chemistry, University of Groningen, The Netherlands).

Results

Acidic phospholipids are required for solubilization of a functional branched-chain amino acid transport system

Protein–lipid interactions of the branched-chain amino acid (Bca) transport protein have been studied with isolated membrane vesicles of *Lactococcus lactis* fused with liposomes by a freeze/thaw-sonication method (hybrid membranes) [1–3]. The activity of the Bca carrier is dependent on the phospholipid composition of the lipid vesicles and functions well in a natural phospholipid mixture derived from *E. coli* membranes. PE is the activating lipid species in this complex mixture [16]. A high activity of Δp-driven leucine uptake is observed in hybrid membranes obtained from membrane vesicles fused with liposomes containing *E. coli* PL/egg PC (1 : 1, mol/mol) (Fig. 1A). The same phospholipid mixture was used for the solubilization of *L. lactis* membrane vesicles and the reconstitution of extracted proteins into liposomes (proteoliposomes). The initial transport rate of both Δp-driven L-leucine uptake (Fig. 1A) and leucine counterflow uptake (Fig. 1B) was two-fold higher in proteoliposomes compared to hybrid membranes. The recovery of membrane proteins with the solubilization/reconstitution method was about 59 ± 5%. Due to the protein losses the phospholipid/protein ratio of proteoliposomes was about 17 : 1 (w/w) compared to 10 : 1 (w/w) for hybrid membranes. The trapped volume of proteoliposomes and hybrid membranes was 17.2 and 7.1 μl/mg of protein, respectively. These results show that the solubilization/reconstitution method can be employed with complete recovery of the activity of the Bca carrier.

One of the main problems in reconstituting membrane proteins is loss of activity during solubilization [7,8]. This inactivation can be prevented by the inclusion of phospholipids during solubilization. The leucine transport system was solubilized with phospholipids present in either the solubilization step, the reconstitution step or both. The final lipid composition and phospholipid/protein ratio was kept constant. In the absence of *E. coli* PL/egg PC (1 : 1, mol/mol) during solubilization hardly any transport activity was recovered in the proteoliposomes. High transport activities were observed only when phospholipids were present during the solubilization step (Fig. 2A). The phospholipid requirement of solubilization/reconstitution steps was further investigated by adding *E. coli* PL in the solubilization and Egg PC in the reconstitution step. In another experiment the order of additions was reversed. The final lipid composition of the proteoliposomes was the same for both preparations. Recovery of transport activity was found to be strictly dependent on the presence of *E. coli* PL in the solubilization step. Addition of *E. coli* PL in the reconstitution step

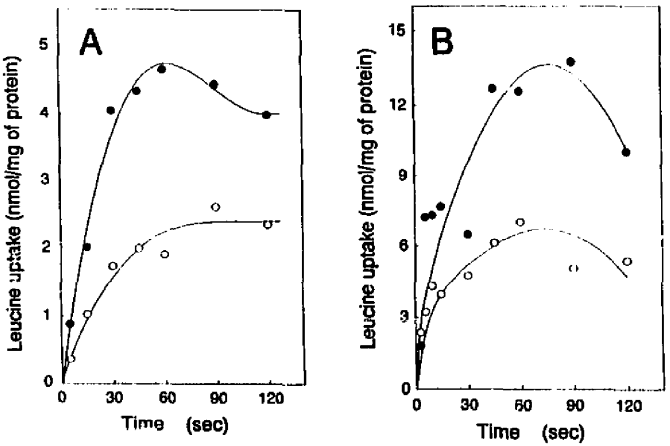

Fig. 1. Δp-driven leucine transport in hybrid membranes obtained by fusion of *L. lactis* membrane vesicles with liposomes (○) or by solubilization and reconstitution of the membrane vesicles (●). Leucine uptake upon the imposition of a Δp (Panel A) or via counterflow (Panel B) was measured in proteoliposomes composed of *E. coli* PL/egg PC (1 : 1, mol/mol).

could not rescue transport activity suggesting that inactivation is an irreversible process (Fig. 2B). These experiments demonstrate that recovery of activity not only depends on the presence of lipids during the solubilization step, but also on the nature of the lipids.

High transport activities have been observed in hybrid membranes of *L. lactis* in which the natural phospholipid mixture is replaced by synthetic phospholipids i.e. DOPE/DOPC (1:1, mol/mol) [16,17]. In contrast, when the same lipid mixture was used in the solubilization step hardly any transport activity was observed (Table I). To establish the nature of the missing lipid component, *E. coli* phospholipids were analyzed on two-dimensional TLC. The commercially obtained *E. coli* lipids contained PE (72 mol%), lyso-PE (5.2 mol%) and CL (20.5 mol%). This phospholipid composition differs from the reported composition [29] in which PE (75–80 mol%) and PG (20 mol%) are the major components, while CL is present only in trace amounts (1–5 mol%). The presence of the relatively high amounts of CL in the commercial preparation could be due to the isolation of the lipids from cells harvested in the late stationary phase in which CL has been accumulated at the expense of PG [30]. The effects of both CL and PG on the recovery of transport activity were tested. *E. coli* PL was used in the reconstitution to ensure activity of the transport systems. When CL was present during solubilization the reconstituted transport activity was 76.1% of the activity obtained when *E. coli* PL/egg PC (1:1, mol/mol) was used in both the solubilization and the reconstitution step (control value) (Table I). The precursor of CL biosynthesis, PG, showed similar activating effects.

TABLE I

Initial rate of Δp-driven leucine uptake in proteoliposomes obtained after solubilization and reconstitution of L. lactis membrane vesicles

Lipid used for solubilization [a]	reconstitution [a]	% of control
–	EcoPL/eggPC (1:1)	3.1
EcoPL/eggPC (1:1)	EcoPL/eggPC (1:1)	100.0
DOPE/DOPC (1:1)	EcoPL	3.5
CL	EcoPL	76.1
DOPG	EcoPL	73.1
DOPG/DOPC (3:1)	EcoPL	156.1
DOPG/DOPC (1:1)	EcoPL	38.1
DOPG/DoPC (1:3)	EcoPL	10.0
DOPC	EcoPL	7.0
DOPG	DOPG	6.7
DOPC	DOPC	6.7
DOPS/DOPC (1:1)	EcoPL	164.2
PS/DOPC (1:1)	EcoPL	67.4
PI/DOPC (1:1)	EcoPL	96.8
L. lactis lipid	EcoPL	88.4
MGDG/DOPC (1:1)	EcoPL	14.7
DGDG/DOPC (1:1)	EcoPL	23.7

[a] Lipid composition in molar ratio.

Since synthetic CL is not commercially available, DOPG was used for further experiments. The effect of the presence of DOPG during solubilization was further analyzed by varying the DOPG content in mixtures of DOPG and DOPC. When the DOPG concentration (and CL, not shown) of the solubilization mix-

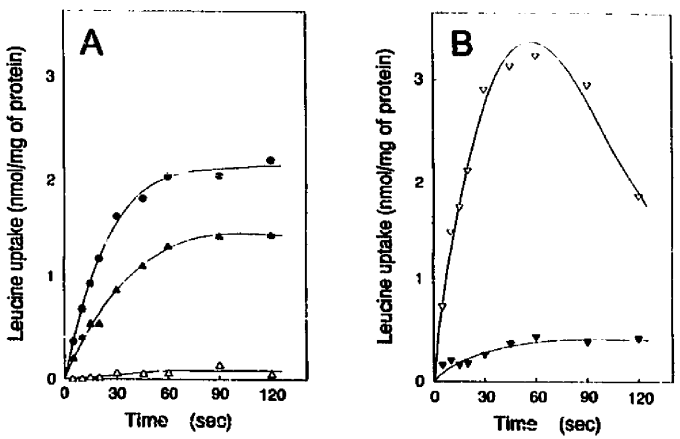

Fig. 2. Δp-driven leucine transport in proteoliposomes obtained by solubilization and reconstitution of *L. lactis* membrane vesicles. Leucine uptake upon the imposition of a Δp was measured in proteoliposomes composed of *E. coli* PL/egg PC (1:1, mol/mol). (Panel A) Effect of *E. coli* PL/egg PC (1:1, mol/mol) added in the solubilization (▲) or the reconstitution step (△) or in both steps (●). (Panel B) Effect of the nature of added phospholipid. *E. coli* PL added in the solubilization and egg PC in the reconstitution step (▽), egg PC in the solubilization and *E. coli* PL in the reconstitution step (▲). Each of proteoliposome preparations had a final lipid/protein ratio of about 20:1 (w/w).

TABLE II

Initial rate of leucine counterflow in proteoliposomes obtained after solubilization and reconstitution of L. lactis membrane vesicles

Lipid used for		V_{in} [b]	% of control
solubilization [a]	reconstitution [a]		
EcoPL/eggPC (1:1)	EcoPL/eggPC (1:1)	32.4	100.0
DOPC	EcoPL	16.2	50.0
DOPE/DOPC (1:1)	EcoPL	8.4	25.9
DOPG/DOPC (1:1)	EcoPL	38.8	119.8

[a] Lipid composition in molar ratio.
[b] V_{in} in nmol/mg of protein per min.

ture decreased, a lower level of transport activity was recovered (Table I). This effect can be ascribed to the negatively charged DOPG, as DOPC alone shows very low transport activity. Table I also reveals that the use of DOPG in the reconstitution step leads to an very low activity of the Bca carrier. The acidic phospholipids PS and PI yielded similar results as DOPG and CL (Table I). The higher activity of a mixture of DOPS/DOPC (1:1, mol/mol) compared to DOPG/DOPC (1:1, mol/mol) can be explained by the activating effect of the amino-PL [16]. A drastically higher transport activity was obtained when the synthetic DOPS instead of the natural bovine brain PS was used in the solubilization step. This difference was also found for hybrid membranes and is most likely due to differences in the acyl chain composition. Although the main fatty acid acyl chain C-number of DOPS and bovine brain PS are about the same (96% C_{18} for bovine brain PS), the saturation index of natural PS is only 50% [31]. Lipids extracted from *L. lactis* membranes contain CL, PG and glycolipids [16], and support the reconstituted leucine transport activity when present during the solubilization (Table I). The recovery of activity by this lipid mixture appears not to be due to neutral glycolipids since MGDG and DGDG present during solubilization yield low transport activities. However, these glycolipids have been found to support leucine transport activity in hybrid membranes [16].

TABLE III

Initial rate of arginine / ornithine exchange in proteoliposomes obtained after solubilization and reconstitution of L. lactis or E. coli membrane vesicles

Bacterium	Lipid used for		V_{in} [b]	% of control
	solubilization [a]	reconstitution [a]		
L. lactis	EcoPL/eggPC (1:1)	EcoPL/eggPC (1:1)	1.82	100.0
	DOPC	EcoPL	0.38	20.9
	DOPE/DOPC (1:1)	EcoPL	0.32	17.6
	DOPG/DOPC (1:1)	EcoPL	2.25	123.6
	DOPG	EcoPL	4.92	270.6
E. coli [c]	EcoPL/eggPC (1:1)	EcoPL/eggPC (1:1)	2.52	100.0
	DOPC	EcoPL	0.75	29.8
	DOPG/DOPC (1:1)	EcoPL	2.52	100.0
	DOPG	EcoPL	4.74	188.1

[a] Lipid composition in molar ratio.
[b] V_{in} in nmol/mg of protein per min measured at 5°C.
[c] The transport system from *Ps. aeruginosa* was cloned in *E. coli*.

The effect of phospholipids present during solubilization was further investigated on leucine counterflow activity (Table II). During counterflow the carrier remains protonated and transport activity is not dependent on the magnitude of Δp. Although less pronounced than found for Δp-driven uptake the highest leucine counterflow activities were also observed when acidic phospholipids were present during solubilization. It is concluded that acidic phospholipids are needed for functional solubilization of the Bca carrier.

Acidic phospholipids are required for solubilization of arginine / ornithine exchangers

To investigate the phospholipid requirement of other transport proteins during solubilization, arginine/ornithine antiporters of *L. lactis* [32] and *Pseudomonas aeruginosa* were reconstituted into proteoliposomes. The latter transport system was extracted from membranes derived from *E. coli* cells in which the *arcD* gene coding for the *Ps. aeruginosa* arginine/ornithine exchanger was expressed (Verhoogt et al. [38]). Optimal recovery of this transport activity required a three-fold higher concentration of phospholipid in the solubilization step compared to extraction conditions of the Bca carrier (Verhoogt et al. [38]). Arginine uptake was performed at 5°C to allow an accurate measurement of the initial transport rate. Recovery of transport activity of both arginine/ornithine exchangers in proteoliposomes required the presence of acidic phospholipid (i.e. PG) in the solubilization step (Table III).

Discussion

The phospholipid dependency of the transport system for branched-chain amino acids (Bca) of *Lactococcus lactis* has been studied using two different *in vitro* systems. Isolated membrane vesicles have been fused with liposomes with the freeze/thaw-sonication procedure. These studies revealed that both the phospholipid headgroup and the fatty acid acyl chain composition are important in determining the transport activity. We now have used a reconstitution procedure in which membrane proteins were first extracted from the

membrane with detergent and subsequently reconstituted into proteoliposomes. The general method for the reconstitution of membrane proteins was first developed by Baron and Thompson [4] and Racker and co-workers [5] who noted that octyl glucoside is a convenient detergent for reconstitution by detergent-dilution. This method was refined by Newman and Wilson [8] who found that addition of phospholipid during solubilization is important for recovery of activity. Ambudkar and Maloney [6] introduced the use of glycerol as a protein stabilizer in the solubilization step. The latter procedure has been successfully used for the functional reconstitution of ion-linked antiport [7] and binding protein-dependent transport systems [33,34], ion-translocating ATPases [7] and the precursor protein translocator of *E. coli* [11]. This study shows that the method can be improved further by controlling the lipid composition during the solubilization step. Recovery of leucine transport activity was found to be strictly dependent on the presence of acidic phospholipids during the solubilization step. This lipid requirement is satisfied by the complex mixture of *E. coli* PL, *E. coli* CL, bovine liver PI, bovine brain PS, and synthetic PG or PS. Although not needed during solubilization, aminophospholipids (or glycolipids) have to be present in the final proteoliposomes for activation of the leucine transport system (see Table I). Those lipids can be added in the reconstitution step. It is important to emphasize that the acidic phospholipid requirement of the leucine transport system during solubilization is not specific for the leucine transport system since two other transport proteins, i.e. arginine/ornithine exchangers originating from two different sources show a similar dependency. Several mitochondrial inner membrane transport proteins have been found to be dependent on CL for activity. This lipid has both a stabilizing and a stimulating effect and is routinely used for the solubilization and/or isolation of those carriers (for Review, see Ref. 35).

The need for acidic phospholipids was not noticed in our previous studies in which the fusion method was employed [16,17]. In the fusion procedure membrane proteins are not subjected to delipidation and detergent extraction. The activities of the transport systems are in membrane fusion only affected by the bulk of the lipid environment since the endogenous phospholipids remain present in the hybrid membranes. The main difference between the two procedures might be related to alterations of the lipid-annulus which surrounds the carrier. In the fusion procedure the lipid-annulus is not disturbed so that the protein can maintain an active conformation. This is evident from experiments in which the inactive carrier in membranes fused with liposomes composed of PC can be reactivated by re-fusion with PE containing liposomes [16]. On the other hand in the solubilization/reconstitution procedure (acidic) phospholipids will be removed which leads to an inactive conformation and the inactivated carrier cannot be reactivated by the addition of PE during the reconstitution step (See Fig. 2). The acidic phospholipids during solubilization will prevent this delipidation and stabilize an active conformation of the carrier. The data suggests that the acidic phospholipid fraction present in the natural membrane is not sufficient. Since *E. coli* PL have often been used for solubilization and/or reconstitution an absolute requirement for acidic phospholipids was not noted with the lactose carrier of *E. coli* [12,13] nor for other transport systems [14,15]. At least for the lactose carrier of *E. coli* this is not due to the experimental conditions employed. Using a mixture of DOPE/DOPC (1:1, mol/mol) during the solubilization of *E. coli* T184/pGM21 [36] membrane vesicles, the lactose permease retained its active conformation upon reconstitution (data not shown). This finding is consistent with published data [12,13]. Another explanation for the discrepancy in results for the different transport proteins could be found in the relative strength by which the lipid-annulus is bound around these proteins.

The lipid requirement in the solubilization step appears to be more important for Δp-driven leucine uptake then for leucine counterflow uptake. Both uptake modes involve the binding of the substrate, but accumulation driven by a Δp takes place only when also a proton is transported. The proton translocation could be more severely influenced by the conformation of the membrane protein. A similar observation has been reported by Young and co-workers [37] who noticed that one function of the lactose carrier of *E. coli* (sugar recognition and translocation, i.e. counterflow) shows a broad tolerance for various phospholipids while cation coupling, i.e. Δp-driven uptake, requires a very defined lipid environment.

We assume that the solubilization/reconstitution method leads to a perturbation of the lipid annulus and a more controlled environment than in the fusion method is required to maintain transport systems active. The finding that the acidic phospholipids have to be present during the solubilization for the recovery of transport activities presents a further improvement of the solubilization/reconstitution procedure for membrane proteins. The procedure could also be beneficial when membrane proteins have to be purified and reconstituted into liposomes of a defined (synthetic) lipid composition.

Acknowledgements

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