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Biochimica et Biophysica Acta 1463 (2000) 407–418

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Properties of a reconstituted eukaryotic hexose/proton symporter solubilized by structurally related non-ionic detergents: specific requirement of phosphatidylcholine for permease stability

Ingrid Robl [a], Renate Graßl [a], Widmar Tanner [a], Miroslava Opekarová [b,*]

[a] *Universität Regensburg, Lehrstuhl für Zellbiologie und Pflanzenphysiologie, Universitätsstr. 31, 93053 Regensburg, Germany*
[b] *Institute of Microbiology, Czech Academy of Sciences, Vídenská 1083,142 20 Prague 4, Czech Republic*

Received 30 August 1999; received in revised form 20 October 1999; accepted 16 November 1999

Abstract

Overexpression of the hexose/proton symporter HUP1 from *Chlorella kessleri* in *S. cerevisiae* permits a one-step purification via a biotinylation domain. Milligram amounts of the protein are obtained starting from 2 l of yeast culture. The HUP1 protein is used as a model eukaryotic membrane protein of the 'major facilitator superfamily' (MFS) to study specific lipid requirements for activity and stability. Testing two series of detergents revealed that *n*-nonyl-β-D-glucoside (NG) and *n*-octyl-β-D-glucoside (OG) solubilize the HUP1 protein efficiently. Only the use of NG resulted in long-term stabilization of the HUP1 protein in the absence of external lipids. When affinity purified protein was extracted with organic solvents, a stoichiometric amount of phosphatidyl choline, phosphatidyl ethanolamine and ergosterol in the ratio of close to 2:1 was detected. These lipids were only observed, however, when the protein purification was carried out in the presence of NG; no lipids were copurified with the HUP1 protein in the presence of OG. Of the three lipids copurified, phosphatidyl choline showed a crucial role in ensuring maximal HUP1 permease activity and stability when added back to the OG-protein. The requirement of phosphatidylcholine documents a specific effect of lipids on vectorial transport mediated by a eukaryotic membrane protein of the MFS family.

Keywords: Hexose/proton cotransporter; Phosphatidylcholine; Nonyl-glucoside; *Chlorella kessleri*

1. Introduction

Integral membrane proteins accomplish their role in a highly organized environment of a lipid bilayer. Effects of different lipids on different classes of integral membrane proteins have been studied both in terms of the physico-chemical state of the lipid bilayer, and of specific effects of individual lipids on the catalytic functions of membrane proteins. Recently, the latter aspect has received increasing attention. Two principal approaches are being employed in this type of study. One line of evidence on specific

Abbreviations: MFS, major facilitator superfamily; NG, *n*-nonyl-β-D-glucoside; OG, *n*-octyl-β-D-glucoside; DM, dodecyl-maltoside; TPP^+, tetra[^{3}H] phenylphosphoniumbromide; TLC, thin-layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PL, crude *Escherichia coli* phosphatidylethanolamine; PAGE, polyacrylamide gel electrophoresis; FTS, freeze/thaw/sonication

* Corresponding author. Fax: +420-2-472-2257;
E-mail: opekaro@biomed.cas.cz

0005-2736/00/$ – see front matter
PII: S0005-2736(99)00229-1

functions of phospholipids at the molecular level has emerged from genetic manipulation, leading to elimination of specific phospholipids in *Escherichia coli* [1]. Altering the membrane composition or eliminating a minor or major phospholipid species can, however, result in a complex set of pleiotropic effects. The other approach, in vitro reconstitution, can more directly contribute to the elucidation of the role of lipids in the activity of membrane bound proteins. Purification of a single membrane protein and its re-insertion into liposomes with defined lipid composition represents a simple system in which lipid effects can be directly measured apart from possible interfering factors found in intact cells. The prerequisite for such studies is a release of the integral protein from its native membrane in an active state allowing its re-incorporation into liposomes. A number of different surfactants are now available for membrane solubilization [2]. Although the behavior of a simple detergent/solvent system can be predicted to some degree from theory, practice shows that purification of each membrane protein and preserving its optimal activity requires an individual experimental design. To understand why some detergents are more suitable for preserving the activity of a membrane protein than others opens one possible way for specifying the lipid requirements of individual proteins.

Work carried out on non-structural functions of phospholipids in eukaryotes has involved almost exclusively mammalian systems. Ca^{2+}-ATPase from sarcoplasmic reticulum [3,4] and P-glycoprotein multidrug transporter [5] are the two proteins most intensively studied in this respect. There exists evidence that, in addition to bulk and annular lipids, the presence of a small number of 'special' phospholipids which can be considered as 'cofactors' interacting specifically with certain sites on the proteins, modulates their activities.

We used the affinity purified hexose uptake protein encoded by the HUP1 gene from *Chlorella kessleri* [6] as a model to study specific lipid requirements of a eukaryotic membrane transporter. The HUP1 protein shows high homology to, e.g., the human glucose transporters [7], and thus to the large MFS family of substrate transporters [8]. The results obtained are therefore most likely of general importance.

Several limiting factors hamper these types of studies: (a) specific permeases are usually present in plasma membranes in min amounts not sufficient for reconstitution studies; (b) during solubilization and purification procedures, the integral membrane proteins are prone to inactivation. This can often be prevented by an addition of external lipids, which, however, may interfere with the final goal to investigate the role of lipids; and (c) in all cases, where the activity of the reconstituted transport protein depends on energization, the involvement of an independent energy-generating system is required which, like the tested permease, may respond to changes in lipid composition.

We report on a heterologous overexpression and affinity purification of HUP1 permease in *Saccharomyces cerevisiae* – a system allowing the isolation of milligram amounts of the membrane protein under laboratory conditions. In a detergent screening study, *n*-nonyl-β-D-glucoside was found to be an 'ideal' detergent preserving high activity of HUP1 permease during its solubilization, purification, and several-days' storage at 4°C. This activity preservation is a prerequisite not only for optimization of functional assays, but also for potential structural analysis of the protein. In addition, analysis of lipids copurified with nonyl-β-D-glucoside-purified HUP1 protein led to the identification of an essential role of phosphatidylcholine in stabilization of this hexose/proton symporter.

2. Materials and methods

2.1. Materials

D-[U-^{14}C] glucose (295 mCi/mmol), tetra[^{3}H]-phenylphosphoniumbromide (TPP^{+}) (24 Ci/mmol), 3-*O*-methyl-D-[U-^{14}C]glucose (106 mCi/mmol), Western blotting reagents, streptavidin-peroxidase-conjugate, and Hybond nitrocellulose were from Amersham Buchler, Braunschweig (Germany). Glucoside and maltoside detergent kits and *n*-nonyl-β-D-glucopyranoside were from Anatrace, Maumee (USA); D(+)-biotin and dodecylmaltoside were from Biomol, Hamburg (Germany); *n*-octyl-β-D-glucoside was from Calbiochem, La Jolla (USA). ImmunoPure-Immobilized Monomeric Avidin was from Pierce,

Rockford (USA). Cellulose-acetate filters (0.2 μm) were from Schleicher and Schuell, Dassel (Germany), TLC aluminum sheets (Silica gel 60) were from Merck, Darmstadt (Germany); crude phosphatidylethanolamine from *E. coli* (type IX), lipids and other chemicals of the highest purity available were from Sigma, Deisenhofen (Germany); polystyrene beads (BioBeads SM2) were from Bio-Rad, Richmond (USA); creatinase was from Roche, Penzberg, (Germany).

2.2. Strains and plasmids

Saccharomyces cerevisiae RE 700A (MATa; hxt1Δ::HIS3::Δhxt4 hxt5::LEU2 hxt2Δ::HIS3 hxt3Δ::LEU2::Δhxt6 hxt7::HIS3) was obtained from Eckhard Boles, Düsseldorf [9]; pNEV E from Norbert Sauer and Jürgen Stolz, Erlangen [10]. *S. cerevisiae* SEY6210 (MATα, ura3-52, leu2-3, 112, his3-Δ200, trp1-Δ901, ade2-101, suc2-Δ9, GAL (ATCC96100)

2.3. Construction of pNEV E HUP1-Bio-His6 and its expression in S. cerevisiae RE 700A and SEY6210

HUP1 fused with the *Klebsiella pneumoniae* biotin acceptor domain and six histidine codons (HUP1-Bio-His6) in pUC 18 constructed by Caspari et al. [11] was ligated into *Eco*RI sites of NEV E [10]. The plasmids with correctly oriented insert were used for transformation of glucose uptake deficient *S. cerevisiae* RE 700A lacking 7 inherent glucose transporters and wild-type yeast SEY6210.

2.4. Isolation of total membranes and plasma membrane fractions from S. cerevisiae

The transformed yeast strain RE700A was grown on YPD to an A_{578} of 10–15. After pelletting and washing, the cells were disintegrated and total membranes and, if needed, plasma membrane fractions were prepared essentially as described by Goffeau and Dufour [12]. During all the steps, the protease inhibitors *p*-phenylmethyl sulfonyl fluoride and 4-aminobenzamidine dihydrochloride were added to a final concentration of 1 and 2.5 mM, respectively. The aliquots of membranes were frozen in liquid nitrogen and stored at −80°C.

2.5. Solubilization and purification of the HUP1 protein

Crude membrane fraction (1 mg membrane protein/ml) from *S. cerevisiae* overexpressing HUP1-Bio-His6 was incubated on ice for 30 min in 50 mM potassium phosphate buffer, pH 6.3, with or without 0.12% *E. coli* phospholipid and the detergents as indicated in individual experiments. Insoluble material was removed by centrifugation (160 000 × *g*, 40 min, 4°C). Aliquots of supernatants and sediments were tested for the amount of biotinylated HUP1 protein as described below. Total supernatants were loaded onto monomeric avidin column equilibrated by corresponding solubilization buffer, and the biotinylated HUP1 protein was purified according to the protocol of Pierce. Aliquots of purified protein were either used immediately or frozen in liquid nitrogen and kept at −80°C until use. The amount of purified protein was estimated either by silver staining on 10% SDS-gel with creatinase as a standard, or, for comparative purposes, by Western blot analysis with streptavidin-peroxidase conjugate.

2.6. Reconstitution of HUP1 protein into proteoliposomes

Known amounts of purified HUP1 protein were incorporated into liposomes consisting of washed *E. coli* phosphatidylethanolamine together with cytochrome *c* oxidase prepared from beef heart mitochondria essentially as described previously [11]. As a rule, to 0.5–1 μg of purified HUP1 protein in 0.1 ml of solubilization buffer, 0.9 ml of solubilized *E. coli* phospholipid (1.2 mg/ml) was added together with 4.4 pmol of cytochrome *c* oxidase. After 10 min on ice, alkylglucoside detergents were removed by dialysis against 1000-fold volume of 50 mM potassium phosphate buffer/2 mM $MgSO_4$, pH 6.3, at 4°C for 15–18 h. Dodecylmaltoside was removed by Bio-Beads SM2 as described in the protocol of Bio-Rad. After detergent removal, the dialysates were frozen in liquid nitrogen, thawed at room tempera-

ture and sonified by probe sonifier (Cole-Palmer Instruments, Chicago, IL) for 3–5 s. The resulting unilamellar proteoliposomes were collected by centrifugation for 40 min at 160 000×*g* at 4°C and resuspended in 300 μl of 50 mM potassium phosphate buffer/2 mM $MgSO_4$, pH 6.3.

2.7. Measurement of membrane potential formation and HUP1 permease activity in reconstituted proteoliposomes

The proteoliposomes were energized by the addition of the electron-donor system of cytochrome *c* oxidase (20 mM ascorbate, 20 μM cytochrome *c*, 200 μM *N,N,N,N′*-tetramethyl-*p*-phenylenediamine). Measurement of membrane potential was performed as described previously [13]. The activity of HUP1 permease was determined from accumulation of D-[U-^{14}C] glucose under the comparable membrane potential formation in the system. The internal volume of proteoliposomes was calculated from the equilibrium concentration caused by energy-independent glucose accumulation and equalled ∼1 μl/mg of phospholipid.

2.8. Identification and quantification of phospholipids associated with purified HUP1 protein

One to three ml of eluates (containing ∼10 μg of biotinylated protein) obtained after column purification of the protein solubilized by individual detergents without external lipids were extracted by vigorous mixing with 3 ml of chloroform/methanol 2:1. After 5 min centrifugation at 4000×*g*, the aqueous upper phase was discarded and the lower organic phase was washed three times with 3 ml of 10% methanol. The final lipid-containing chloroform phase was evaporated by a stream of nitrogen and the dried lipids were resuspended in 8 μl of toluene. The separation of lipids was achieved by one-dimensional high performance thin-layer chromatography with five step-wise developments as described by Ruiz and Ochoa [14]. For quantification, standard lipids were dissolved in toluene and spotted on TLC plate in the indicated amounts. After TLC, the lipids were charred according to Bitman and Wood [15].

3. Results

3.1. Expression of HUP1 permease in S. cerevisiae

Usually specific permeases are present in plasma membranes in minute amounts. To obtain sufficient material for in vitro studies, the protein of interest is generally overexpressed homo- or heterologously. The HUP1p hexose/proton cotransporter from *Chlorella kessleri* has been functionally expressed in *S. pombe* [16], which made subsequent in vitro and certain in vivo studies possible [11,13,17,18]. However, this heterologous expression, as in many other cases, interfered with the growth of the host organism, and the yield of permease obtained from *S. pombe* was low, therefore.

This situation improved considerably when *S. cerevisiae* strain RE 700 A lacking seven inherent glucose transporters was transformed by the plasmid p NEV E-HUP1-Bio-His6 bearing HUP1 tagged with a biotinylation domain (see Section 2). This modified glucose/proton co-transporter was considerably overexpressed in yeast and the transformed cells were able to grow on D-glucose as sole carbon source. The *Chlorella* HUP1 cDNA functionally fully substituted the missing glucose permeases which originally mediated an energy independent glucose transport (facilitated diffusion) in the wild-type. The overexpression does not interfere with yeast viability, and the heterologous transporter can be efficiently coupled to the protonmotive force of the host organism (Fig. 1). This leads to a 150-fold accumulation of the non-metabolizable D-glucose analog 3-*O*-methyl-glucose in the transformed yeast mutant (Fig. 1A). In transformed *S. cerevisiae* wild-type cells, only a 20-fold accumulation is observed (Fig. 1B), which certainly is due to the continuous loss of accumulated 3-*O*-methyl-D-glucose via the endogenous glucose facilitators. The amount of HUP1 protein expressed in *S. cerevisiae* is virtually independent of growth phase and HUP1 permease can be isolated and affinity purified from solubilized membrane proteins (Fig. 1C). The protein is fully active when incorporated into cytochrome *c* oxidase energized liposomes (see below).

The transformed *S. cerevisiae* mutant could be grown in rich medium; the cells doubled in 90 min

and the culture reached a total density of A_{578} ~15 at stationary phase of growth. Instead of about 10 l of cultivation medium which were necessary to purify 1 mg of HUP1 protein from *S. pombe,* the same amount of the protein can now be obtained from 2 l of *S. cerevisiae* culture.

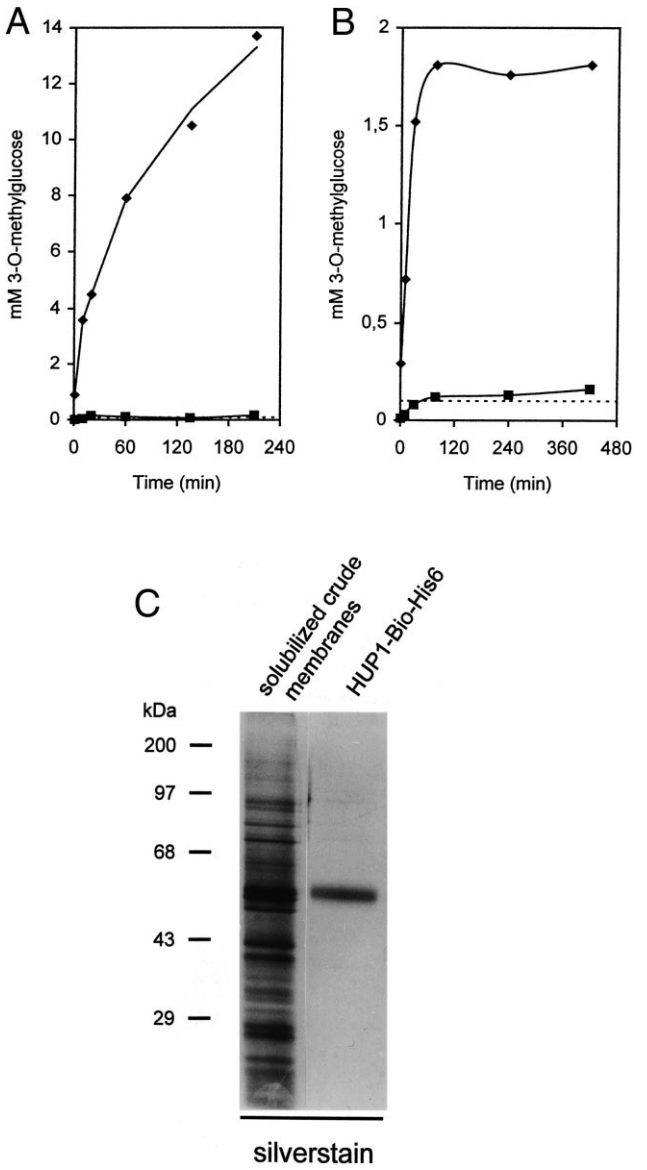

Fig. 1. Accumulation of 3-*O*-methylglucose by transformed *S. cerevisiae* cells lacking seven glucose facilitators (A) or wild-type cells (B). One-step purification of HUP1p from solubilized membrane proteins (C). (A,B) The accumulation of 3-*O*-methylglucose is shown in cells expressing HUP1 (♦) and in cells transformed with the empty vector for control (■). Concentration equilibrium (0.1 mM) is indicated as a dotted line. Sugar uptake in whole cells was measured as described in [16]. (C) HUP1 protein was solubilized with octylglucoside and purified in the presence of *E. coli* phospholipid as described in Section 2 and silver stained as described [11].

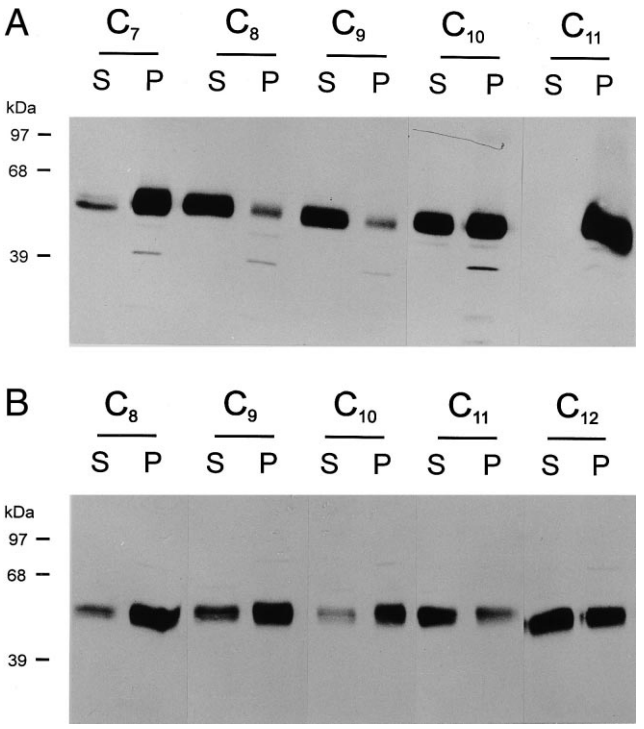

Fig. 2. Solubilization of HUP1p by alkylglucopyranosides and alkylmaltopyranosides differing in the length of aliphatic chains (C_7–C_{12}). Equal amounts of membranes from HUP1 protein overexpressing yeast were solubilized by alkylglucopyranosides (1%, except for C_7 2%) (A) or alkylmaltopyranosides (0.2%, except for C_8 1.25%) (B) as described in Section 2. After ultracentrifugation, the amounts of solubilized and non-solubilized HUP1 protein were estimated in aliquots of supernatants (S) and pellets (P), respectively. Amounts of 10 μg of total protein were subjected to SDS-PAGE and Western blot, and biotinylated HUP1 protein was identified by streptavidin-peroxidase conjugate.

3.2. Solubilization of the HUP1 protein from overexpressing yeast

Obtaining information on structure, function and a potential specific lipid requirement of a membrane protein requires its solubilization and purification. First, a number of detergents have been analyzed to find an optimal one for efficient solubilization of the HUP1 protein and preservation of its activity. Two groups of mild non-ionic, sugar pyranoside-containing detergents, alkylglucosides and alkylmaltosides, differing in the length of their alkyl chains were tested. The solubilization efficiency was esti-

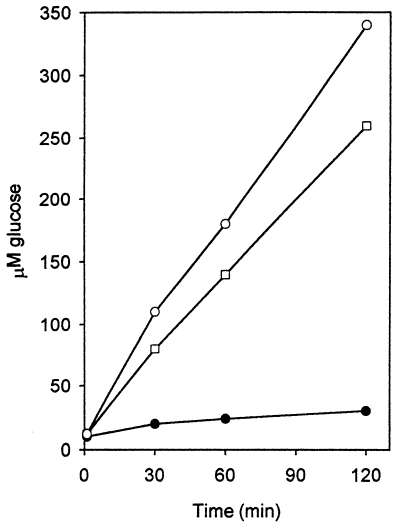

Fig. 3. Accumulation of glucose by purified HUP1 protein reconstituted in cytochrome *c* oxidase containing proteoliposomes. Effect of detergent. HUP1 protein was solubilized and purified by octyl-β-D-glucoside (○), nonyl-β-D-glucoside (□), or dodecylmaltoside (●) in the presence of 0.12% of *E. coli* phospholipid. External concentration of ^{14}C-labelled glucose was 20 µM. The accumulation was started by addition of electron donor system as described in Section 2. The magnitude of membrane potential measured for each individual curve ranged from −128 to −133 mV.

mated from the amounts of HUP1 protein in high-speed centrifugation supernatant (solubilized protein) and pellet (non-solubilized protein). To maintain the solubilized protein in dispersion, the minimum surfactant concentration required is always in excess of the critical micellar concentration (CMC). In the first screening, the detergents were therefore used at concentrations higher than their respective CMC. As documented in Fig. 2, the amounts of solubilized protein differed for each detergent. The most efficient detergents from alkylglucosides, *n*-octyl-β-D-glucoside, *n*-nonyl-β-D-glucoside, and dodecylmaltoside, well solubilizing and widely used in other membrane protein purifications, were used for further studies. To minimize their possible harmful effects, we determined the lowest concentrations sufficient for solubilizing the protein with the same efficiency as above. The optimal concentrations for OG and DM were the same as those used in the first screening (1 and 0.2%, respectively). The concentration of NG could be decreased to 0.3% without lowering the yield of solubilization (data not shown).

3.3. *Effect of different detergents on the activity of purified HUP1 permease*

As previously found, the presence of 0.12% crude *E. coli* phosphatidylethanolamine preserved the HUP1 permease activity during its solubilization and purification with *n*-octyl-β-D-glucoside [11]. Therefore, the same conditions were used to compare the ability of the three detergents (OG, NG, DM) to keep the purified protein in a functional state. Equal amounts of the HUP1 protein solubilized and purified in the presence of the individual detergents were subjected to dialysis (OG- and NG-purified protein) or to BioBeads (DM-purified protein) to remove the detergents. Before removal of the detergent cytochrome *c* oxidase was added as described in Section 2. The reconstitution process in the vesicles can often be improved by introducing a freeze–thaw–sonication step (FTS). The proteoliposomes formed were therefore subjected to FTS and collected by centrifugation.

The activity of HUP1 permease was tested by monitoring the uptake of D-[^{14}C]glucose into the resulting vesicles. Since D-glucose accumulation de-

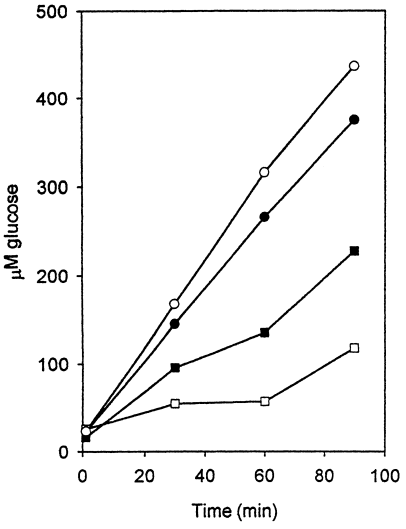

Fig. 4. Accumulation of glucose by HUP1 protein purified without external lipid in cytochrome *c* oxidase containing proteoliposomes. HUP1 protein was solubilized by octyl-β-D-glucoside (□,■) or nonyl-β-D-glucoside (○,●) and purified in the absence of any external lipid. The reconstitution to 0.12% of *E. coli* phospholipid was accomplished either immediately after the purification (■,●) or after a 3-hour incubation in an ice-bath (○,□). The uptake conditions were as in Fig. 3. The magnitude of membrane potential ranged from −134 to −139 mV.

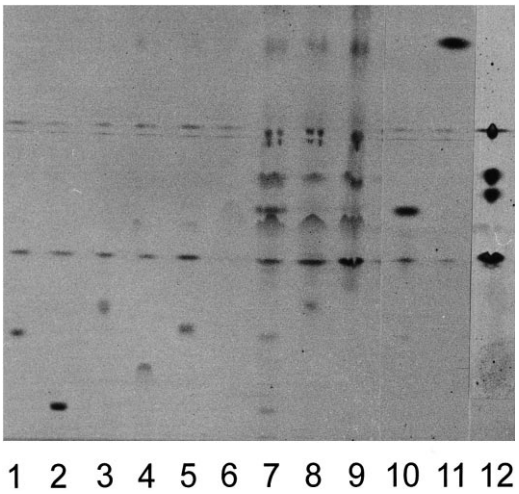

Fig. 5. Representative thin-layer chromatogram of lipids copurified with HUP1 permease by three different alkylglucosides. Lipids extracted from ~10 μg of HUP1 protein purified in *n*-nonyl-β-D-glucoside (lane 7), dodecylmaltoside (lane 8), and octyl-β-D-glucoside (lane 9), were dissolved in 8 μl of toluene and spotted on TLC plate. Lipid standards PE, PC, PG, PS, cardiolipin, and stearic acid, in amounts of 0.5 μg each (1.5 μg for PC) were spotted in lanes 1, 2, 3, 4, 5, 6, respectively. In lanes 10, 11, and 12, ergosterol (0.5 μg), cholesterylester (0.5 μg), and a mixture of mono-, di- and triglycerides (~10 μg) were applied. The plate was developed and the lipids were charred according to the procedure referred to in Section 2. From the amounts of the lipid standards used, it was estimated that 10 μg of NG-purified HUP1 protein contained approximately 0.2 μg of PE, PC and ergosterol.

pends on the actual amount of HUP1 protein incorporated into the vesicles as well as on the magnitude of the proton motive force (pmf) formed in the system, both parameters were checked for each transport experiment. Membrane potential is the main pmf component driving the D-glucose accumulation in this in vitro system [13]. Its magnitude was used as a measure of energization of the system and its value is given in each individual figure. D-glucose accumulation observed in the proteoliposomes containing HUP1 protein solubilized and purified with different detergents is shown in Fig. 3. It is evident that solubilization by DM resulted in an inactivation of the permease, whereas the detergents containing the glucopyranoside (OG, NG) preserved its activity to about the same extent. To exclude the possibility that the different way of detergent removal might affect the protein activity, the OG-purified protein was also reconstituted by detergent removal with BioBeads and found to be active (data not shown). Further investigations were, therefore, carried out with OG- and NG-solubilized HUP1 protein.

The transport activity measured in Fig. 3 has been carried out with HUP1 permease purified in the presence of an excess of crude *E. coli* phosphatidylethanolamine (ratio of HUP1 protein:total phospholipid ≈1:30). Since this large amount of external lipid is unfavorable for any further structural studies (e.g. 2D-crystallization trials) as well as for investigating a potential specific lipid requirement of the permease, we tried to purify the membrane protein without any addition of external lipids. The HUP1 protein solubilized and purified by OG and NG in the absence of external lipids was reconstituted into crude *E. coli* phosphatidylethanolamine by the standard procedure (see Section 2). Omission of external lipids during the preparation of the permease did not affect the activity of NG-purified protein, but resulted in a reduction of activity when OG was used for purification (Fig. 4). Moreover, the protein solubilized and purified in the presence of OG but in the absence of external lipids was gradually inactivated during prolonged storage at 4°C, whereas the activity of NG-purified HUP1 protein remained constant (Fig. 4).

To find an explanation for this difference in behavior, it was assumed that different detergents may delipidate membrane proteins to a varying extent. It has been shown repeatedly that delipidation of membrane proteins by detergents often results in their complete and irreversible inactivation.

3.4. Identification of lipids copurified with HUP1 protein solubilized by different detergents

The acquisition of sufficient amounts of purified protein enabled us to see whether lipids at all copurify with the membrane protein in dependence on the different detergents used in this study. For this purpose we solubilized the membrane fraction from overexpressing yeast and purified it by OG, NG or DM in the absence of external lipids. The amounts of HUP1 protein purified by individual detergents were estimated by Western blot analysis or by silver staining and volumes containing equal amounts of protein (~10 μg protein) were dialyzed to remove the detergents. The lipids retained with the purified protein were extracted and identified on TLC as described in Section 2. The spots visualized after stain-

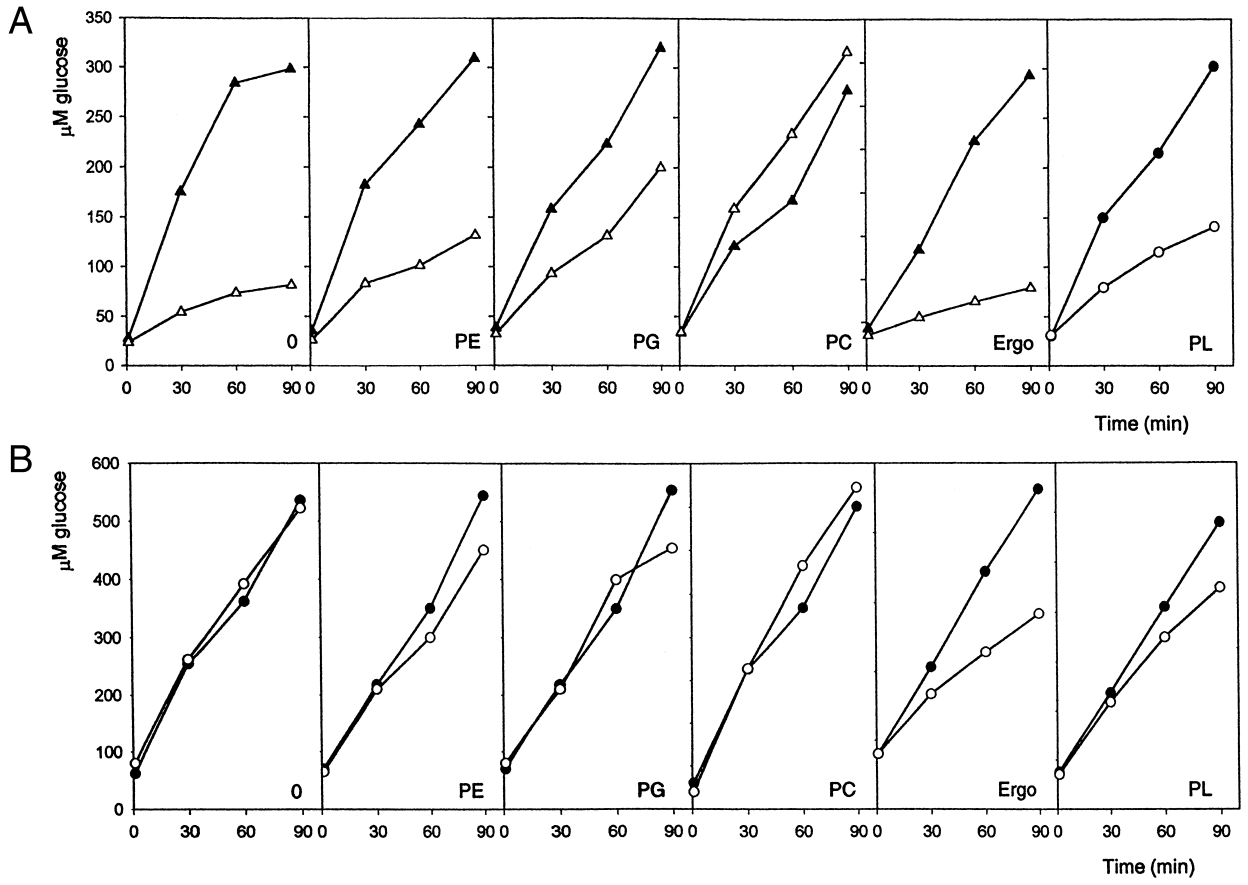

Fig. 6. Protection of HUP1 protein against activity loss during storage on ice. HUP1 protein was solubilized and purified by *n*-octyl-β-D-glucoside (A) or *n*-nonyl-β-D-glucoside (B) in the absence of any external lipid. Two parallel samples were tested for the effect of each individual lipid. One µg of each indicated lipid was added to 100 µl aliquots of the final column eluate (~1 µg of purified HUP1 protein); 0 = control without any external lipid. One of each parallel was reconstituted to 0.12% of *E. coli* phospholipid immediately (closed symbols), the other one after a 4-hour incubation on ice (open symbols). The glucose uptake was measured as in Fig. 3 and is expressed as intravesicular concentration. Membrane potential magnitude measured for each individual curve ranged from −132 to −136 mV.

ing were assigned to lipid standards. As shown in Fig. 5, two phospholipids and one sterol (phosphatidylcholine (PC), phosphatidylethanolamine (PE) and ergosterol) could be clearly detected in lane 7, where NG-purified HUP1 protein was analyzed. Some ergosterol, but virtually no PE and PC, could be detected in extracts from DM- and OG-purified proteins (lanes 8 and 9, respectively). The spots at the top of the plate are present in all three samples and probably represent ergosteryl-ester (see lane 11, cholesteryl-ester standard). The spots second from top found in all lanes including standards, were not identified. Spots present to different degrees in all three samples extracted from HUP1 protein (lanes 7–9) correspond by their mobility to mono-, di- and triglycerides (lane 12); the smeary patch immediately below the ergosterol resembles by shape and mobility free fatty acids (lane 6, stearic acid). Finally, the spot found only in lane 8 was identified on a different plate as DM which could not be removed by dialysis. Quantification of lipid/protein stoichiometry in NG-purified protein revealed that approximately two or three molecules of each phospholipid or ergosterol, respectively, copurified together with one molecule of HUP1 protein (see legend of Fig. 5).

3.5. Protection of HUP1 permease activity by individual lipids

The above results documented that the activity of

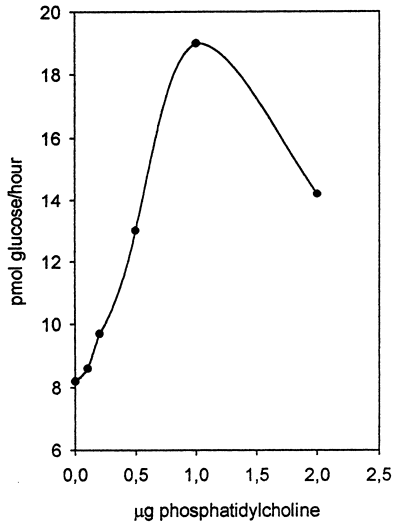

Fig. 7. Concentration dependence of specific protection of HUP1p activity by phosphatidylcholine. Aliquots of octyl-β-D-glucoside-purified HUP1 protein containing ~1 µg protein were protected against activity loss during storage on ice-bath by addition of different amounts of phosphatidylcholine (from egg yolk, ~99% pure). After 4 h, HUP1 protein was reconstituted by the standard method described in Section 2. The uptake of D-glucose was measured as in Fig. 3. The membrane potential ranged from −128 to −131 mV.

the HUP1 protein was best preserved when NG was used for its solubilization and purification. Since only in the presence of NG the protein copurified with PE, PC and ergosterol, these compounds may be the reason for the increased activity and stability of the permease under this condition. To see whether all three components or any one of them may be responsible for the protective effect, we checked whether the inactivation of the permease could be prevented by the addition of individual pure lipids to essentially lipid-free OG-purified HUP1 protein. To 0.1 ml of purified protein (~1 µg), 1 µg of each lipid was added. The samples were left on ice for 4 h and then reconstituted into *E. coli* phospholipid by the standard procedure. Parallel samples for each lipid were reconstituted immediately (within 10 min after HUP1 protein purification). As control, the same set of experiments was performed with NG-solubilized and purified HUP1 protein. One representative set of data of three independent experiments is shown in Fig. 6A,B. As already documented in Fig. 4, the NG-purified protein neither lost its activity during 'aging' on ice, nor was it affected by the addition of any tested lipid (Fig. 6B). From all the lipids tested, the inactivation of OG-purified protein could be prevented only by the addition of PC (Fig. 6A). The protecting effect of PC was concentration dependent and exhibited a maximum at about 1 µg of PC added to approximately 1 µg of protein (Fig. 7). Based on a calculation of the lipid/protein ratio, about 100–200 molecules of PC per one molecule of HUP1 protein afforded the best protection.

4. Discussion

For reconstitution of integral membrane proteins into functional proteoliposomes or for trials to produce crystals of such proteins amenable to structural analysis, the protein of interest has to be obtained in pure form and in sufficient amounts. For the *Chlorella* HUP1 protein, this has been achieved by heterologous overexpression of HUP1 cDNA in the plasma membrane of *S. cerevisiae*. The overexpression reported here enabled us to purify to homogeneity about 1 mg of HUP1 permease from 1 l of yeast culture.

One of the limiting factors in obtaining information on functional aspects of membrane proteins is related to the difficulty of having reproducible methods of reconstitution at hand, including the choice of a detergent and the purification conditions. In general, non-ionic detergents are less effective in dissociating protein complexes, but many proteins are more stable in non-ionic detergents than in ionic ones. In our screening of solubilizing abilities of two series of sugar pyranoside-containing detergents (Fig. 2A,B), *n*-octyl-β-D-glucoside, *n*-nonyl-β-D-glucoside and dodecylmaltoside were found to be the most efficient ones. OG and DM represent the detergents most widely used for solubilizing a broad range of functionally active membrane proteins. To our knowledge, NG has not yet been used in reconstitution studies. In a study of the permeability of phosphatidylcholine liposomes, its properties were found to be similar to those of *n*-octyl-β-D-glucoside [19]. The long-term stability of a membrane protein in native conformation in the absence of external lipids reported in this study makes NG a good candidate for the use in two-dimensional crystallization studies.

NG thus might be suitable for purification and reconstitution of a range of other transporters.

It has been repeatedly demonstrated that preservation of membrane protein activity requires the presence of a certain amount of external lipids. Indeed, in the presence of 0.12% *E. coli* phospholipid during solubilization and purification steps, HUP1 protein purified by OG and NG exhibited high activity when reconstituted into proteoliposomes. In contrast, the treatment by DM resulted in an irreversible HUP1 permease inactivation (Fig. 3). This finding is surprising because this detergent has been widely used in solubilization of many diverse active membrane proteins. OG, NG and DM molecules feature an intermediate length of the hydrophobic moiety and a bulky hydrophilic sugar-head group. The reported common mechanism of OG- and DM-mediated reconstitution of different membrane proteins [20] has been attributed to the common structure of these two detergents, i.e. a glycosylated polar head group [21]. Proper folding of a membrane protein into the lipid bilayer is a prerequisite for its activity and is determined to a large degree by the matching of the protein's hydrophobic parts to the thickness of the lipid bilayer. Under our conditions using crude *E. coli* phosphatidylethanolamine as the bulk lipid in which the HUP1 protein has been reconstituted, the thickness of the membrane was the same in all cases.

The method and velocity of detergent removal might affect both the folding of the protein and the homogeneity and unilamellarity of the proteoliposomes. Unlike OG and NG, DM cannot be removed by dialysis due to its low critical micelle concentration of 0.009%. Detergent removal by BioBeads used as an alternative in this study, resulted in a formation of sealed proteoliposomes (formation of membrane potential of about −135 mV), but the HUP1 protein incorporated into the vesicles was inactive (Fig. 3). The same procedure used for reconstituting OG-purified protein, however, yielded an active protein, indicating that the method of detergent removal is not decisive for preservation of HUP1 permease activity. Possible differences in the unilamellarity of proteoliposomes formed under different conditions were eliminated by including a FTS step in both cases.

Two possible reasons may be considered to explain different effects of the three detergents on the permease activity. (1) The preservation of the protein activity in the presence of these detergents with a glucose ring as headgroup might be envisaged as a substrate-mediated stabilization of the permease. Indeed, in a study of the nature of the glucose binding pocket of the renal sodium/D-glucose cotransporter, it was reported that the β-anomers of these two alkylglucosides at low subsolubilizing concentrations specifically inhibited glucose transport in brush border membrane vesicles. Kinetic analysis revealed a fully competitive type of inhibition, indicating that the polar head group of these detergents binds to the substrate recognizing site [22]. The more bulky head of DM might not fit the D-glucose pocket of HUP1 permease and thus the possible protein stabilization of hexose transporter is precluded. (2) Complete delipidation of a membrane protein generally results in its inactivation. This is often prevented by including an excess of external lipids during the solubilization/purification procedure. In our study, the omission of external lipids resulted in gradual time-dependent inactivation of OG-purified HUP1 protein, whereas it did not affect the activity of the permease purified by NG (Fig. 4). The aliphatic chains of these detergents differ only by one $-CH_2$ group. It can be speculated that the longer NG hydrophobic moiety can better mimic fatty acyl chains of yeast plasma membrane (10–20 carbons) and thus provide a better stabilization of the protein. We did not check this possibility, since detergents with longer hydrophobic chains exhibited decreased solubilizing effects (Fig. 2A). A possible explanation for the preservation of HUP1 permease activity could consist in the different amount and pattern of lipids remaining attached to the protein molecule when solubilized by different detergents, a feature not studied so far to our knowledge.

The pattern of lipids copurified with the HUP1 protein using NG, OG or DM revealed that only NG-purified protein is accompanied by PE, PC, and ergosterol (Fig. 5). These lipids do not reflect the yeast total membrane composition which comprises in addition PI, PS, sphingolipids and mitochondrial PG and cardiolipin [23,24]. We calculated that only two or three molecules of PC, PE and ergosterol, were co-purified with one molecule of the protein in the presence of NG. Since about 50 lipid molecules would be necessary to surround the

biotinylated protein (~68 kDa) in a monomolecular layer [25], the two or three molecules cannot represent total annular lipids. Even these small amounts, however, protect NG-purified protein from its time-dependent inactivation observed with lipid-free OG-purified HUP1 protein. It can be shown that this property can be attributed specifically to PC (Fig. 6A). The protection of HUP1 protein activity depends on the amount of PC available in the assay. The best protection against time-dependent inactivation was provided at a PC/HUP1 protein ratio of about 100–200. Tentatively, the protective effect of two or three PC molecules found associated with NG-purified protein could be attributed to their specific binding at certain sites of the membrane-spanning parts of the permease, resulting in a stabilization of the protein conformation. Once this specifically bound PC is removed, e.g. by OG treatment, the protein may become more prone to unfolding. For probability reasons and because of the possibly low reversibility of the process, the restoration of the PC-HUP1p by exogenously added PC might require a higher PC concentration (Fig. 7).

Increasing attention has recently been paid to lipids acting as 'cofactors' of membrane proteins and affecting thus their activities. Besides some direct evidence emerging from crystallographic studies [26,27], and the rigorous elucidation of the crucial role of PE in the *E. coli* lac permease assembly and function [1], documentation of a specific role of individual lipids on the activity of integral membrane proteins is still scarce.

In this respect, the P-glycoprotein multidrug transporter and Ca^{2+}-ATPase from sarcoplasmic reticulum are the most extensively studied eukaryotic membrane proteins. Thermal stabilization of P-glycoprotein [28] was observed with lipid concentrations two to three orders of magnitude higher than that found here for the HUP1 permease. While HUP1 protein activity was specifically preserved by PC and exhibited a maximum at about 1μg PC/ml of final reconstitution mixture, P-glycoprotein activity was protected by dipalmitoyl-phosphatidylethanolamine, PS and partially by PI, at lipid concentration of about 0.3 mg/ml and higher. Three major phospholipids, PE, PC and PI, were identified to be associated with P-glycoprotein. Based on the quantification of 53–56 phospholipid molecules which would be sufficient to completely surround the membrane spanning domains of the protein, they probably represent annular lipids [5]. The activity of Ca^{2+}-ATPase from sarcoplasmic reticulum was doubled by phosphatidylinositol 4-phosphate interacting specifically with certain sites of the protein [29,30]. Similarly, evidence for specific cholesterol binding sites was reported [31]. These studies documented the lipid effects on only one step (usually a non-vectorial one) in the complex activities of these transporters, e.g. ATP splitting and/or substrate binding, while they did not include the actual vectorial process of substrate translocation. The specific requirement of minute amounts of PC reported in our study is, to our knowledge, the first report demonstrating a specific lipid effect on vectorial transport mediated by a eukaryotic membrane protein.

Acknowledgements

This work was supported by Grant Agency of Czech Republic (204/99/0492), by the Deutsche Forschungsgemeinschaft (SFB 521) and by a NATO Collaborative Research Grant (LST.CLG 974804)

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