Unidirectional Reconstitution into Detergent-destabilized Liposomes of the Purified Lactose Transport System of Streptococcus thermophilus*

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The lactose transport protein (LacS) of Streptococcus thermophilus was amplified to levels as high as 8 and 30% of total membrane protein in Escherichia coli and S. thermophilus, respectively. In both organisms the protein was functional and the expression levels were highest with the streptococcal lacS promoter. Also a LacS deletion mutant, lacking the carboxy-terminal regulatory domain, could be amplified to levels >20% of membrane protein. Membranes from S. thermophilus proved to be superior in terms of efficient solubilization and ease and extent of purification of LacS; >95% of LacS was solubilized with relatively low concentrations of Triton X-100, n-octyl-β-D-glucoside, n-dodecyl-β-D-maltoside, or C12E8. The LacS protein carrying a poly-histidine tag was purified in large quantities (~5 mg/liter of culture) and with a purity >98% in a two-step process involving nickel chelate affinity and anion exchange chromatography. The membrane reconstitution of LacS was studied systematically by stepwise solubilization of preformed liposomes, prepared from E. coli phospholipid and phosphatidylcholine, and protein incorporation at the different stages of liposome solubilization. The detergents were removed by adsorption onto polystyrene beads and H⁺-lactose symport and lactose counterflow were measured. Highest transport activities were obtained when Triton X-100 was used throughout the solubilization/purification procedure, whereas activity was lost irreversibly with n-octyl-β-D-glucoside. For reconstitutions mediated by n-dodecyl-β-D-maltoside, C12E8, and to a lesser extent Triton X-100, the highest transport activities were obtained when the liposomes were titrated with low amounts of detergent (onset of liposome solubilization). Importantly, under these conditions proteoliposomes were obtained in which LacS was reconstituted in an inside-out orientation, as suggested by the outside labeling of a single cysteine mutant with a membrane impermeable biotin-maleimide. The results are consistent with a mechanism of reconstitution in which the hydrophilic regions of LacS prevent a random insertion of the protein into the membrane. Consistent with the in vivo lactose/galactose exchange catalyzed by the LacS protein, the maximal rate of lactose counterflow was almost 2 orders of magnitude higher than that of H⁺-lactose symport.

The lactose transport protein (LacS) of Streptococcus thermophilus is a hybrid protein composed of a polytopic membrane domain that is predicted to span the cytoplasmic membrane 12 times and a carboxy-terminal cytoplasmic domain of about 180 amino acids (Poolman et al., 1989). The hydrophilic domain is homologous to IIA protein(s) (domains) of various phosphoenolpyruvate-sugar phosphotransferase systems and is involved in the regulation of this secondary transport system (Poolman et al., 1995b). The hydrophobic carrier domain, which is sufficient for transport activity, is homologous to a new family of secondary transporters to which belong the melibiose transport proteins (MelB) of Escherichia coli, Klebsiella pneumoniae, and Salmonella typhimurium, the glucuronide transport protein (GusB) of E. coli, the xylose transport protein (XylP) of Lactobacillus plantarum, and various other proteins that have not been studied in great detail (Poolman and Konings, 1993; Poolman et al., 1996).

The LacS protein of S. thermophilus has been functionally expressed in E. coli and was shown to catalyze the transport of a variety of α- and β-galactosides. Studies in membrane vesicles from S. thermophilus fused with cytochrome c oxidase containing liposomes showed that the sugars are accumulated by a H⁺-solute symport mechanism driven by both a membrane potential and a pH gradient. The uptake of lactose (precursor) can also be driven by a coupled exchange reaction with galactose (product) without the net movement of protons, which reflects the prominent transport reaction in vivo (Poolman, 1990; Foucaud and Poolman, 1992).

In order to elucidate the structure of the protein and the regulation of its activity it was necessary to purify the protein and to develop methods for efficient and unidirectional reconstitution into artificial membranes. Previously, the expression levels in E. coli were too low to identify the LacS protein on Coomassie-stained SDS-PAGE1 gels. In the present study, different expression systems based on different promoters, ex-

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1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; sp (or pmf), proton motive force; bp, base pair(s); C12E8, octaethylene glycol monododecylether; CMC, critical micelle concentration; CSPD, disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2-octaethylene glycol-4,10}dicyclohexyl)phosphate; MPB, 3-(N-maleimidylpropionyl)-biocytin; NTA, nitrilotriacetic acid; PL, phospholipids; PC, phosphatidylcholine; Pipes, 1,4-piperazinediethanesulfonic acid; TMG, methyl-1-thio-β-D-galactopyranoside.
Reconstitution of the Lactose Transport System of S. thermophilus

Table I
Plasmids used

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant properties</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSK111(+)</td>
<td>Carb9, high copy expression vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pSK8E</td>
<td>pSK111(+), carrying lacS of S. thermophilus A147 as 4073-bp EcoRI fragment (galM', lacS')</td>
<td>Poolman et al. (1992)</td>
</tr>
<tr>
<td>pSK8E8</td>
<td>pSK8E with EcoRI site 21-bp upstream of the initiation ATG of lacS</td>
<td>This work</td>
</tr>
<tr>
<td>pSK8E8M1u</td>
<td>pSK8E with MluI site on the stop codon of lacS</td>
<td>This work</td>
</tr>
<tr>
<td>pSK8E8His</td>
<td>pSK8E with his-tagged lacS</td>
<td>This work</td>
</tr>
<tr>
<td>pTAQI</td>
<td>Carb9, pBR322, lac behind penicillin promoter, medium copy number vector, inducible tac promoter</td>
<td>Genencor Int., San Francisco</td>
</tr>
<tr>
<td>pLS1</td>
<td>pTAQI, carrying 165-bp EcoRI-Dral fragment from pSK8E lacS of S. thermophilus</td>
<td>This work</td>
</tr>
<tr>
<td>pLLO1</td>
<td>pLLO1, carrying 165-bp EcoRI-Dral fragment from pSK8E lacS of S. thermophilus</td>
<td>This work</td>
</tr>
<tr>
<td>pMTC15</td>
<td>Carb9, high copy number expression vector, inducible tac promoter</td>
<td>Footnote 2</td>
</tr>
<tr>
<td>pSK8E8N</td>
<td>pSK8E with Ncol site on the initiation codon of lacS</td>
<td>This work</td>
</tr>
<tr>
<td>pLLO11</td>
<td>pMTC15 with galP (1404-bp Ncol-HindIII fragment) replaced by 2225-bp Ncol-EcoRI fragment of pSK8E</td>
<td>This work</td>
</tr>
<tr>
<td>pGK13</td>
<td>Cm9, Em9, E. coli, S. thermophilus shuttle vector</td>
<td>Kok et al. (1984)</td>
</tr>
<tr>
<td>pGKG58</td>
<td>pGK13, carrying lacS of S. thermophilus as 3824-bp EcoRI-Dral fragment from pSK8E ligated into the EcoRI-EcoRV sites</td>
<td>Poolman et al. (1995b)</td>
</tr>
<tr>
<td>pGK58(lacΔ160)</td>
<td>pGKGS8 with 480-bp deletion of IIA domain</td>
<td>This work</td>
</tr>
<tr>
<td>pGKH1</td>
<td>pGKG8 with his-tagged lacS</td>
<td>This work</td>
</tr>
<tr>
<td>pSK8E(C320A)</td>
<td>pSK8E with cysteine 320 of LacS replaced by alanine</td>
<td>This work</td>
</tr>
<tr>
<td>pGKH2(C320A)</td>
<td>pGKH2 with cysteine 320 replaced by alanine</td>
<td>This work</td>
</tr>
<tr>
<td>pGKH3(C320A,S384C)</td>
<td>pGKH3 with serine 384 replaced by cysteine</td>
<td>This work</td>
</tr>
</tbody>
</table>

Plasmid Constructions

The plasmids used for the expression in E. coli and S. thermophilus and their relevant properties are listed in Table I and oligonucleotides are listed in Table II. For the subcloning of the lacS gene with its own ribosome binding site, a new EcoRI restriction site was engineered by directed mutagenesis. Single-stranded uracil-containing DNA of plasmid pSK8E was isolated from E. coli C236 and served as a template for the annealing of the oligonucleotide primer LacSpd2. Closed-circular DNA was synthesized in vitro as described (Kunkel et al., 1987) and the resulting plasmid (pSK8E) was transformed into E. coli MC1061. The new EcoRI restriction site was checked by restriction analysis and by nucleotide sequencing of double stranded DNA using the deoxy chain termination method (Sanger et al., 1977). The 165-bp EcoRI-Dral fragment of plasmid pSK8E was isolated and ligated into the EcoRI and Smal sites of the medium copy number vector pTAQI, yielding plasmid pLS1; the same fragment in the high copy vector pTEXT1, yielding plasmid pLLO1. In both plasmids the gene is under control of the isopropyl-β-D-thiogalactopyranoside inducible tac promoter. For the expression of LacS from the galP promoter in plasmid pMTC15, a Ncol site was engineered into plasmid pSK8E that overlaps with the initiation codon of lacS (primer NoCa) and a second Ncol site present in the coding region was removed without changing the amino acid sequence (primer NcoB). The resulting plasmid pSK8EBN was checked by nucleotide sequencing as described. After digesting plasmid pSK8EBN with EcoRI and treatment with Klenow enzyme the DNA was digested with Ncol and the 2225-bp Ncol-blunt end fragment was isolated. Plasmid pMTC15 was partially digested with HindIII and the linearized plasmid was treated with Klenow enzyme. Subsequently, the linearized plasmid was isolated and digested with Ncol and the 3315-bp NcoI

Bacterial Strains and Growth Conditions

E. coli strains used were HB101 (hsdS20[r des-m'] M, recA3, ara14, proA2, lacY1, galK2, rps [Sm9], xy5, mtl1, supE44, α- F-). MC1061 (ala[POZY], araD139, [ara-leu]7697, galU, galK, rpsL, rmbL, NO2947 (MC1061, rexA56 strr1::Tn10), and C236 (dut, ung, thi, relA, srl, ung, thi, relA, ung) (1956) supplemented with 0.5% beef extract, 20 μM lactose, and with 5 μM erythromycin when carrying plasmid pGK13 or derivatives (Poolman et al., 1995b).
blunt end fragment was ligated with the 2225-bp Ncol-blunt end fragment of pSK8N. The resulting plasmid was termed pLL11.

### Engineering of His-6-tag to Wild Type LacS

Site-directed mutagenesis was used to generate a unique Mlu restriction site (ACCCGGT) that overlaps with the stop codon of lacS gene. Single-stranded uracil-containing DNA of plasmid pSK8E was isolated from E. coli Cj 236 and served as a template for the annealing of oligonucleotide primer MluI. The resulting plasmid (pSK8EMlu) was transformed to E. coli MC1061 and the presence of the new MluI site was checked by restriction analysis. Subsequently, a linker, consisting of the two annealed oligonucleotides His-link A and His-link B, that codes for a factor Xa deavage site and a poly(G)histidine tag, was ligated in the MluI site of pSK8EMlu. The insert and flanking regions were checked by nucleotide sequencing as described. The unique BstEII and DraI sites in the flanking regions were used to isolate a 224-bp fragment, coding for the carboxyl terminus with the His-6-tag, and this fragment was exchanged with the wild type fragment from pSK8E, yielding pSK8BHs.

For expression of the His-tagged protein in S. thermophilus, the 3824-bp EcoRI-DraI fragment of pSK8EHis was isolated and ligated in the unique EcoRI and EcoRV sites of shuttle vector pGK13 (Kok et al., 1984), yielding plasmid pGKHis. S. thermophilus strains were transformed as described (Mallet et al., 1993).

### Construction of Cysteine Mutants of LacS-H6

A gene coding for a LacS protein devoid of cysteine residues (wild type contains one cysteine) was made by site-directed mutagenesis using single stranded DNA of plasmid pSK8E as a template and oligonucleotide C320A as primer. The resulting plasmid was termed pSK8BC320A. For expression of the mutant protein in S. thermophilus, the 2874-bp EcoRI-PstI fragment of plasmid pSK8EBC320A was isolated and ligated in the EcoRI and PstI sites of pGKHis, thereby replacing the wild type fragment and yielding plasmid pGKH(C320A). Using a two-step polymerase chain reaction approach the serine at position 384 was replaced by a cysteine in the Cys-less LacS-H6. Oligonucleotides S384C and pEXG (Table II) were used to amplify a 810-bp fragment of pSK8E, which, subsequently, was used as a primer in the second polymerase chain reaction with oligonucleotide EcoRI and Pfblcl site of pGKH(C320A). The expected 1295-bp fragment was digested with PstI and KpnI and ligated into the PstI and KpnI sites of pGKH(C320A), thereby replacing the wild type fragment and yielding pGKH(C320A/S384C). The 551-bp PstI-KpnI fragment of the mutant was checked by nucleotide sequencing.

### Isolation of Membranes

For the isolation of right-side out membrane vesicles of E. coli, cells were disrupted by water lysis as described (Witholt and Boekhout, 1978). For the isolation of inside-out membrane vesicles, cells were lysed by passage through a French pressure cell (20,000 p.s.i.) (Poolman et al., 1983).

Right-side out membrane vesicles of S. thermophilus were isolated as described (Otto et al., 1982) with the following modifications: the cell wall was digested with 10 mg/ml lysozyme plus 10 units/ml mutanase; DNase and RNase were added to final concentrations of 100 μg/ml each. To remove peripheral membrane proteins as well as cytosolic contaminants, membrane vesicles were extracted with 5% urea and 6% (w/v) sodium cholate as described (Newman et al., 1981). The membrane preparations were stored in liquid nitrogen.

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### Solubilization

Membranes (3-4 mg of protein/ml) were solubilized in 50 mM potassium phosphate, containing 20% (v/v) glycerol. Optimal solubilization conditions were determined for the different detergents with respect to pH, NaCl and detergent concentration (see "Results"). The suspensions were mixed and after 10-20 min of incubation at 4°C, the insoluble material was removed by centrifugation (280,000 × g, 15 min). The amounts of LacS in the soluble and insoluble fractions were quantitated by Coomassie staining of SDS-PAGE gels and immunoblotting. Conditions routinely used to compare the solubilization efficiency of membranes from E. coli and S. thermophilus involved the use of the following medium: 50 mM potassium phosphate, pH 8.0, 100 mM NaCl, 20% (v/v) glycerol plus detergent.

### Immunoblotting

The amount of wild type and LacS-H6 in the different samples was estimated by immunodetection with antibodies raised against synthetic peptides and directed against the NH2 or COOH terminus of the wild type protein (Poolman et al., 1995b). The membrane proteins were separated by SDS-PAGE (12.5% polyacrylamide) and transferred to polyvinylidene difluoride membranes by semi-dry electrophoretic blotting. Detection, using the Western-LightTM chemiluminescence detection kit with CSPD™ as a substrate, was performed as recommended by the manufacturer (Tropix Inc.).

### Purification of LacS-H6

The solubilized membrane proteins were mixed and incubated for 30 min with Ni-NTA resin (~4 mg of LacS/ml of resin) that was equilibrated with buffer A (50 mM potassium phosphate, pH 8.0, 100 mM NaCl, 10% (v/v) glycerol, 0.2 mg/ml E. coli lipids plus detergent at a concentration of 5 × CMC) plus 10 mg/ml NiCl2. The column material was poured into a Bio-Spin® column (Bio-Rad) and washed with 10 column volumes of buffer A containing 25 mM imidazole. The protein was eluted with buffer A, pH 7, containing 200 mM imidazole. The purification was carried out at 4°C with sterile solutions. Fractions from the Ni column were diluted 10 times with buffer B (50 mM potassium phosphate, pH 7, 10% (v/v) glycerol, 0.2 mg/ml E. coli lipids plus detergent at a concentration of 5 × CMC) and loaded onto a Mono Q column (HR 5/5, Pharmacia Biotech). The proteins were eluted with a NaCl gradient running from 0 to 750 mM in buffer B.

### Reconstitution of LacS-H6

For the reconstitution of purified protein, liposomes were prepared from acetone/diethyl ether washed E. coli lipids and L-α-phosphatidylcholine from egg yolk in a ratio of 3:1 (w/w). Unilamellar vesicles with relatively homogenous size were made by dialysis of octyl glucoside dissolved lipids (Driessen and Konings, 1993), followed by freezing in liquid nitrogen, slow thawing at room temperature, and extrusion through a 400-nm polycarbonate filter (Mayer et al., 1986). Subsequently, the liposomes were diluted to 4 mg of PL/ml and titrated with detergent, which was followed by measuring the absorbance at 540 nm using a SLM-Aminco spectrophotometer (Paternostre et al., 1988). The liposomes titrated with detergent were mixed with the purified protein in a ~1:100:1 ratio (w/w), unless specified otherwise, and incubated for 30 min at 20°C under gentle agitation. For the removal of detergent, polylysine beads (Bio-Beads SM-2; extensively washed with methanol and H2O) were added at a wet weight of 80 mg/ml (liposomes suspension of 4 mg of PL/ml) and the sample was incubated for another 2 h at room temperature. Fresh polylysine beads were added twice, after removal of Bio-Beads by filtration on glass silk, and the samples were incubated.
at 4 °C for 3 h and overnight, respectively. The proteoliposomes were washed with 50 mM potassium phosphate, pH 7.0, harvested by centrifugation, and stored in liquid nitrogen.

Orientation of the Reconstituted LacS Protein

The mutant proteins LacS(C320A) and LacS(C320A/S384C) were expressed in S. thermophilus and purified using Triton X-100, and reconstituted into liposomes titrated with a low concentration (1.8 mM) of the detergent. Proteoliposomes were washed twice and resuspended to a final protein concentration of ~0.5 mg/ml in potassium phosphate, pH 7.5, 100 mM NaCl, and labeled from the outside with 300 μM MPB at 30 °C. For the outside and inside labeling, the proteoliposome samples were labeled following sonication with a microtip at an output of 4 μm (peak to peak) for 3 s in the presence of MPB (Bayer et al., 1985). The labeling reactions were stopped by the addition of 10 mM dithiothreitol, and analyzed by Western blotting and detection with streptavidin/alkaline phosphatase and CSPD™ as a substrate.

Transport Assays

(i) Lactose Uptake in E. coli—Cells were grown overnight in Luria broth, washed, and resuspended in KPM (50 mM potassium phosphate, pH 7.0, plus 2 mM MgSO₄) supplemented with 10 mM l-Lactate. After 2 min of energization in the presence of oxygen, [14C]lactose was added to a final concentration of 50 μM and uptake was assayed for different time intervals. The reaction was stopped by diluting the mixture with 2 ml of ice-cold 0.1 M LiCl and the cells were collected on 0.45-μm cellulose nitrate filters. The filters were subsequently washed with another 2 ml of the LiCl solution.

(ii) TMG Counterflow in S. thermophilus—Cells grown in Beliker broth with 0.5% lactose or 0.5% sucrose were washed and resuspended in KPM. The cells were resuspended with 2 mM TMG for 1 h at 42 °C and concentrated by centrifugation. The concentrated cell suspensions were diluted 40-fold into 100 mM potassium phosphate, pH 6.6, 2 mM MgSO₄, containing [14C]TMG at a final concentration of 57 μM. The reaction was stopped as described above (i).

(iii) Lactose Counterflow in Proteoliposomes—Proteoliposomes were resuspended in KPM plus 10 mM lactose and frozen in 1-ml aliquots in liquid nitrogen. After thawing the samples at room temperature, the liposomes were extruded through a 400-nm filter to obtain unilamellar liposomes. Accumulation of lactose is not detectable in proteoliposomes. The uptakes correlated with the expression levels of LacS in the membranes. The expression in NO2947/pLL01 was about 2-fold lower than in NO2947/pSKE8 (data not shown). The overexpression of the E. coli glucuronide transport protein (GusB) from the tac promoter in pTTQ18 is shown for comparison (Fig. 1, lanes 4 and 8) (~25% of total membrane protein). The initial rate of lactose uptake in whole cells (at 50 μM, final concentration) was 6 nmol/min/mg of protein for NO2947/pSKE8 as compared to 2.6 nmol/min/mg of protein for NO2947/pLL01, which correlate with the expression levels of LacS in the membranes. Accumulation of lactose is not detectable in E. coli NO2947/pTTQ18 (control).

Overexpression of LacS in S. thermophilus—For the amplification of LacS in a homologous expression system, the lacS gene with about 2 kilobases of 5′ DNA from the chromosome of S. thermophilus was ligated into plasmid pGK13, a shuttle vector that replicates in both E. coli and S. thermophilus. The plasmid, pGKS8, was transformed into S. thermophilus ST11 and ST11(lacS) and the cells were grown in Beliker plus lactose as carbon and energy source and to induce the lacS promoter. The transport activity of both strains was compared to strains carrying the plasmid control pGK13 (Fig. 2). TMG counterflow rates were highest in strains containing pGKGS8; the additional copy of the lacS gene present on the chromosome of the wild type ST11 did not lead to a higher transport activity, indicating that the maximum attainable level of expression was reached. It was difficult to determine the initial rates of TMG uptake accurately, but, in various trials, the transport activity of ST11(lacS)/pGKS8 (or ST11/pGKS8) was at least 2-3-fold higher than ST11/pGK13. Similar results were

obtained with LacS-H6 (histidine tag engineered to the COOH terminus) instead of wild type LacS. To quantitate the levels of expression of LacS, right-side out membrane vesicles of S. thermophilus were analyzed by SDS-PAGE before and after extraction with 5 M urea and 6% (w/v) sodium cholate. Fig. 3 shows the expression levels in S. thermophilus of LacS-H6 using pGKGS8 as expression vector. These levels varied between 25 and 30% of total membrane protein in urea/cholate extracted membranes, and were independent of whether the His6-tag was engineered to the COOH terminus of the protein (data not shown). Lanes 1 and 4 show a strain in which the lacS gene was present on the chromosome rather than on the plasmid (the lacS genes of strain A147 and ST11 are identical). The reduced expression level corresponds with the lower activity of TMG counterflow in ST11/pGK13 as compared to ST11(lacS)/pGKGS8 (Fig. 2). Also, the LacS protein without the IIA domain (LacSΔIIA) could be overexpressed to high levels (lanes 3 and 6). LacS-H6 is migrating at a slightly higher molecular weight compared to the wild type as a result of 10 additional amino acids, i.e. IEGRRHHHHHH. Electron microscopy of negatively-stained cells showed that overexpression did not lead to the formation of inclusion bodies either in E. coli or S. thermophilus (data not shown). Moreover, subcellular structures such as stacked membranes or invaginations were not seen in electron micrographs of cells overexpressing LacS.

Solubilization—The detergents octyl glucoside (0.8–2.0%), n-dodecyl-β-D-maltoside (0.1–1.0%), C₁₂E₈ (0.5 to 8%), and Triton X-100 (0.1 to 2%) were tested for their ability to solubilize LacS from S. thermophilus and E. coli membranes. Solubilizations were carried out at pH 6.0–8.0 with NaCl concentrations varying from 0 to 600 mM, and protein concentrations ranging from 1 to 5 mg/ml. The degree of solubilization was similar at every pH value tested (data not shown). Since the protein is subsequently used for purification on a Nickel column, a pH of 8.0 was used in most experiments and the concentration of the protein was kept relatively high at 4 mg/ml (Pourcher et al., 1995). For the detergents used the effect of NaCl was significant; increasing concentrations of NaCl decreased the efficiency of solubilization and the protein seemed to aggregate in the presence of high concentrations of salt (600 mM). This was also observed by SDS-PAGE where the protein runs as higher order aggregates, especially when C₁₂E₈ was used as detergent (data not shown). Irrespective of whether octyl glucoside, n-dodecyl-β-D-maltoside, C₁₂E₈, or Triton X-100 was used, more than 95% of LacS and -60% of total protein were solubilized from membranes of S. thermophilus (Table III). For the comparison of the solubilization of LacS from membranes of E. coli and S. thermophilus, buffer C (50 mM potassium phosphate, pH 8.0, 100 mM NaCl, 20% of glycerol) and total membrane protein concentrations of 4 mg/ml were used. Under these conditions the efficiency of solubilization of both types of membranes was significantly different (Table III). Further testing of solubilization conditions with the membranes from E. coli did not lead to an improvement.

Purification—Since the expression levels of LacS in S. thermophilus were highest and the solubilization was far more efficient than in E. coli, right-side out membranes from S. thermophilus were used as starting material for the purification of the LacS-H6 protein (Fig. 4, lane 1). The His-tagged protein could almost be purified to homogeneity in a single step using nickel chelate affinity chromatography (Fig. 4, lane 3), even when the urea-cholate extraction step was omitted (Fig. 4, lane 2). Most contaminants were removed by washing the column with 25 mM imidazole, at medium ion strength (100 mM NaCl, 50 mM potassium phosphate, pH 7.0, 100 mM NaCl, 10% of glycerol), and peak fractions contained LacS at a concentration of 1.0–1.5 mg/ml and with a purity of >95%. The minor contaminants were also binding to the Ni-affinity resin when a total membrane extract, containing wild type LacS rather than LacS-H6, was applied to the column (data not shown). This indicates that these contaminants are not associated with LacS per se. Further purification was achieved by anion exchange chromatography on a Mono Q column (Fig. 4, lane 4). The protein was eluted at a NaCl concentration of ~500 mM. From 1 liter of cells, grown in Belliker medium plus lactose to an OD₆₀₀ = 0.8–1.0, 40–50 mg of crude membrane protein was obtained, from which 4–5 mg of LacS was purified. The NH₃-terminal sequence of the purified protein was Met-Glu-X-Ser-Lys-Gly-Gln-Met-Lys-Ser-Arg, which is identical to the deduced amino
TABLE III
Percentages of LacS protein solubilized from membranes of E. coli and S. thermophilus using different detergents

<table>
<thead>
<tr>
<th>Detergent</th>
<th>E. coli</th>
<th>S. thermophilus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium cholate (0–6% w/v)</td>
<td>&lt; 5%</td>
<td>&lt; 5%</td>
</tr>
<tr>
<td>Octyl-β-D-glucoside (1% w/v)</td>
<td>&lt; 70%</td>
<td>&gt; 95%</td>
</tr>
<tr>
<td>Dodecyl-β-o-maltoside (0.5% w/v)</td>
<td>&lt; 70%</td>
<td>&gt; 95%</td>
</tr>
<tr>
<td>C12E8 (2% w/v)</td>
<td>&lt; 50%</td>
<td>&gt; 95%</td>
</tr>
<tr>
<td>Triton X-100 (0.5% w/v)</td>
<td>&lt;40%</td>
<td>&gt;95%</td>
</tr>
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</table>

Reconstitution—Detergent-mediated reconstitution of LacS from solubilized membranes of S. thermophilus has been successful using octyl glucoside and detergent removal by dilution or dialysis (Foucaud and Poolman, 1992). However, when LacS protein was purified in the presence of octyl glucoside, no transport activity was observed upon reconstitution into proteoliposomes, using detergent dilution, dialysis, or adsorption to polystyrene beads to remove the octyl glucoside. Also, the first attempts using C12E8 or n-dodecyl-β-D-maltoside gave low transport activities and a low reproducibility. Therefore, the reconstitution process had to be examined more precisely, which was done using strategies described by Rigaud et al. (1988). Since membrane reconstitution is dependent on the type of detergent used, lipid to protein ratios, and the physical state of the lipid/detergent mixture, these parameters were studied in detail. Rather than using dispersed lipids, preformed liposomes formed the starting material for our reconstructions, unless indicated otherwise. The liposomes are composed of E. coli PL and egg PC in a ratio of 3:1 and were formed by detergent dilution, followed by freeze/thaw/extrusion as described under “Experimental Procedures.” Although the lipid requirement of LacS has not been studied in detail, liposomes prepared from mixtures of E. coli PL and egg PC yielded the highest activities as was observed for other transport proteins of (lactic acid) bacteria (Driessen et al., 1988). To follow the physical state of the liposomes the absorbance at 540 nm was measured upon stepwise addition of detergent. At different stages of the titration curve, purified LacS protein was added to the lipid/detergent mixture and then the detergent was removed at a controlled rate with the use of polystyrene beads (Holloway, 1973).

An example of reconstitution of LacS into preformed liposomes at different detergent concentrations is shown for C12E8 (Fig. 5); low, medium, and high correspond to 2.2, 4.1, and 10.1 mM C12E8, respectively. The uptake of lactose was measured in a counterflow assay upon equilibration of the proteoliposomes with lactose and 100-fold dilution into buffer containing [14C]lactose; the final lactose concentration was 60 μM. The assay temperature was 30°C.

Using the same approach, the optimal conditions for reconstitution were also determined for other detergents. n-Dodecyl-β-D-maltoside shows a titration curve in which the absorbance of the liposome suspension is decreasing initially, after which a steep increase in absorbance is observed. Since equilibration of n-dodecyl-β-D-maltoside and liposomes is slow, the solubilization process cannot be analyzed accurately (see also Rigaud and Pitard, 1995). The counterflow activity was again measured at detergent concentrations at which, on the basis of the

Fig. 5. Counterflow activity in proteoliposomes of LacS-H6 purified and reconstituted with C12E8. Purified LacS-H6 was added to preformed liposomes titrated with low (2.2 mM), medium (4.1 mM), and high (10.1 mM) concentrations of C12E8. After removal of detergent, the proteoliposomes were washed with potassium phosphate, pH 7.0, plus 2 mM MgSO4 (KPM), and loaded with 10 mM lactose by freeze/thaw/extrusion. The counterflow reaction was started by diluting concentrated proteoliposome suspensions (~1–5 mg of protein/ml) 200-fold into KPM containing [14C]lactose; the final lactose concentration was 60 μM. The assay temperature was 30°C.
Absorbance changes, the liposomes are predicted to be in the lamellar, the lamellar-micellar state (50% decrease A_{540}), and the micellar state. Fig. 6 shows a similar n-dodecyl-β-D-maltoside concentration dependence of the lactose uptake rate as was observed for C_{12}E_{8} (Figs. 5 and 6). The transport activity was highest at the n-dodecyl-β-D-maltoside concentration at which the liposomes were destabilized but most likely still in the lamellar state. These observations explain the low activities in the first reconstitution experiments with n-dodecyl-β-D-maltoside and C_{12}E_{8}, because these were always performed with mixed micelles of lipid and detergent.

Using octyl glucoside the purified LacS protein could not be functionally reconstituted (Fig. 6). Since LacS can be reconstituted functionally from total membrane protein extracts obtained with octyl glucoside, this detergent seems to inactivate the protein during the purification. The presence of carrier substrates (galactosides) throughout the solubilization/purification/reconstitution did not improve the LacS activity in the proteoliposomes, i.e. when octyl glucoside was used. The highest transport activities were obtained using Triton X-100 as a detergent and initial uptake activities of up to 800 nmol/min/mg of protein were found that depended relatively little on the physical state of the liposomes, i.e. lamellar or micellar (Fig. 6).

The counterflow activities in the proteoliposomes were not only dependent on the physical state of the liposomes at the beginning of reconstitution but also on the glycerol concentration, the NaCl concentration, lipid to protein ratio, temperature, and rate of detergent removal. Glycerol concentrations above 1% (v/v) at the beginning of the reconstitution reduced the counterflow activities of the proteoliposomes; 600 mM NaCl also reduced the activity probably due to aggregation of the protein; lipid to protein ratios (weight/weight) of 100–200 were optimal (data not shown); and higher transport activities were obtained when LacS protein was allowed to insert into the detergent-destabilized liposomes at 20 rather than at 4°C and when the detergent was removed at a controlled rate by adding small amounts of polystyrene beads (~100 mg/ml liposome suspension). Less important seemed to be the pH (between 6 and 8) and the presence of lipids during the solubilization. Reconstitution conditions that gave the highest activity for Triton X-100 solubilized and purified LacS include: elution of the LacS protein from the nickel column in 50 mM potassium phosphate, pH 7.0, 100 mM KAc plus 2 mM MgSO_{4} by freeze/thaw/extrusion. All aliquots of concentrated proteoliposomes were diluted 100-fold into 120 mM NaPipes, pH 7.0, plus 2 mM MgSO_{4}, containing 0.5 μM valinomycin plus [^{14}C]lactose at the concentration indicated. The data were fitted to Michaelis-Menten and replotted as Lineweaver-Burk (inset). The transport activities in the proteoliposomes were not dependent on the physical state of the liposomes, prepared from the purified LacS protein in Triton X-100.

The counterflow assay was performed as described in the legend to Fig. 6. For the Δp-driven lactose uptake reaction, proteoliposomes were loaded with 20 mM potassium phosphate, pH 7.0, 100 mM KAc plus 2 mM MgSO_{4} by freeze/thaw/extrusion. All aliquots of concentrated proteoliposomes were diluted 100-fold into 120 mM NaPipes, pH 7.0, plus 2 mM MgSO_{4}, containing 0.5 μM valinomycin plus [^{14}C]lactose at the concentration indicated. The data were fitted to Michaelis-Menten and replotted as Lineweaver-Burk (inset).
Fig. 8. Labeling of reconstituted LacS(C320A/S384C)-H6 with MPB. Proteoliposomes with LacS(C320A/S384C)-H6 or LacS(C320A)-H6, using 1.5 mg Triton X-100 for the reconstitution, were washed and resuspended in potassium phosphate, pH 7.5. For the outside labeling (shown for LacS(C320A/S384C) and LacS(C320A)), MPB was added to a final concentration of 30 μM and the reaction was performed at 30°C for 0, 10, and 30 min. For inside and outside labeling (only shown for LacS(C320A/S384C)), the samples were sonicated at zero time. Proteins were separated on SDS-PAGE (12.5%) gels, transferred to polyvinylidene difluoride membranes, and the MPB-labeled protein was detected with streptavidin/alkaline phosphatase using CSPD as a substrate.

The lactose transport protein of S. thermophilus (LacS) has been overexpressed using E. coli and S. thermophilus as expression hosts. Surprisingly, the highest expression in E. coli was obtained when the protein was expressed from the lacS promoter and with the streptococcal Shine-Dalgarno sequences. The levels of LacS in membranes of lactose-grown wild type S. thermophilus cells are higher than the highest amplification reached in E. coli. These expression levels could even be increased by expressing the LacS protein from a plasmid carrying the lacS gene behind the lacS promoter (ST11lacS/pGKGS8). When the protein was expressed from the plasmid in a wild type background, with an additional copy of the gene on the chromosome (ST11/pGKGS8), the expression was not increased further. The increase in expression level of LacS in ST11lacS/pGKGS8 relative to ST11/pGK13 conforms with a 2–3-fold increased rate of TMG counterflow. Although the S. thermophilus expression system is preferred for the isolation and purification of wild type and mutant LacS proteins, the levels of expression in E. coli are sufficient to perform the initial screening of mutants and to purify mutant proteins that cannot be expressed in S. thermophilus. Besides the higher expression in S. thermophilus, purification of the LacS protein from this organism has the additional advantage that the membranes of this Gram-positive organism are more efficiently solubilized, with a whole range of detergents, than those of E. coli.

Since the LacS protein could be reconstituted from octyl glucoside solubilized membranes of S. thermophilus (Foucaud and Poolman, 1992), our first attempts to reconstitute the purified protein were performed with octyl glucoside as detergent. Although several reconstitution parameters were varied, we were unable to obtain active proteoliposomes. On the basis of SDS-PAGE, LacS seems to aggregate when purified in octyl glucoside which may prevent functional insertion into the lipid bilayer. It has been suggested that octyl glucoside is able to penetrate into the hydrophobic parts of a membrane protein, thereby partially unfolding and destabilizing the polypeptide (Lund et al., 1989; Rigaud and Pitard, 1995).

To optimize the reconstitution, the strategy of Rigaud et al. (1988) was followed. The liposomes, however, were prepared differently and made from total E. coli phospholipids plus egg PC in a 3:1 ratio. The liposomes were made by freeze/thaw/evaporation to obtain relatively large and homogeneous vesicles. The physical state of the liposomes during the titration with the detergent was followed by measuring the absorbance at 450 nm. Titration curves obtained with Triton X-100 and octyl glucoside are comparable with the curves that have been published for liposomes composed of egg PC/phosphatidic acid in a 9:1 ratio, which were made by reverse-phase evaporation (Paternostre et al., 1988). Titrations with n-dodecyl-β-D-maltoside and C12E8 show a similar behavior as Triton X-100. The increase in A450 is probably reflecting the swelling of the liposomes due to the partitioning of detergent molecules in the membrane, and the subsequent decrease most likely reflects the disintegration of the liposomes. When liposomes are titrated with n-dodecyl-β-D-maltoside, however, the steady state absorbance values are reached very slowly (>30 min), whereas the absorbance changes by the other detergents take place rapidly (<2 min). It is possible that n-dodecyl-β-D-maltoside first binds to the interface region of the liposomes and then slowly integrates into the hydrocarbon region by rearrangement of detergent and lipid molecules. The concentration of octyl glucoside at which the liposomes are destabilizing (decrease in the absorbance) matches with the CMC of the detergent (~25 μM), which corresponds to a molar ratio of lipid to detergent of 1:6. For the detergents with the lower CMC’s, the liposomes start to destabilize when the molar ratio of lipid to detergent is about 1:1. The relative high amounts of octyl glucoside necessary to destabilize the liposomes might also be a factor that negatively affects the functional reconstitution.

DISCUSSION

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Although the structures formed by the lipid/detergent mixtures are hard to predict, the changes in absorbance offer a good diagnostic parameter for optimizing the reconstitution conditions. For C12E8, n-dodecyl-β-D-maltoside and to lesser extent Triton X-100, the highest activities correspond with the maximal $A_{550}$ value, presumably reflecting the saturation of the liposomes with detergent and the transition from the lamellar to the mixed micellar form (onset of solubilization). We speculate that at this point the LacS protein is inserted unidirectionally into the lipid bilayer by fusion of detergent-saturated liposomes and protein/detergent micelles. This fusion/insertion is improved by mixing the liposomes and protein at 20°C rather than 4°C (data not shown), which might be related to the increased fluidity of the lipid bilayer at higher temperatures and/or a change in the micellar molecular weight (Hjelmeland, 1980). Also, the rate of detergent removal is critical, as fast removal of the detergent decreased the transport activity of the proteoliposomes. A low Bio-Bead concentration is particularly important in the first step of the reconstitution in order to avoid loss of phospholipids and to allow the protein to insert into the detergent destabilized membrane. Once the protein is inserted into the bilayer, the Bio-Bead concentration is less critical but needs to be sufficient to remove the residual detergent molecules.

The advantage of reconstituting membrane proteins into detergent destabilized liposomes might be that one has a better control of the incorporation of the protein into the bilayer and that the protein is faced with lower detergent concentrations. In the case of H$^+$-ATPase, Ca$^{2+}$-ATPase, and other proteins (Richard et al., 1990; Levy et al., 1992; Rigaud and Pitard, 1995), it has been observed that a more uniform orientation is obtained when the proteins are reconstituted into preformed liposomes (Eytan, 1982). The large hydrophilic domains of these enzymes may prevent a scrambled orientation, which is usually observed when proteins are reconstituted from a suspension of mixed micelles (Rigaud and Pitard, 1995). A similar situation may hold for the LacS protein, which has a hydrophilic domain of about 180 amino acids at the COOH terminus. It is possible that this hydrophilic N1A domain forms an "anchor" that prevents the protein inserting randomly.

The kinetics of counterflow and of Δp-driven lactose uptake yield the same apparent affinity constants, i.e. approximately 0.17 mM. This is surprising since previous studies in E. coli indicated a $K_{mp}$ for Δp-driven lactose uptake of 0.8 mM, whereas the $K_{mp}$ for non-equilibrium exchange (equivalent to the counterflow activity measured in this study) was estimated to be 10 mM (Poolman et al., 1992, 1995a). It should be stressed that the apparent affinity constants in previous determinations reflect the outside conformation of the protein whereas the present data correspond with the inside conformation of the LacS protein.

The higher $V_{max}$ of the counterflow reaction as compared to the Δp-driven uptake reaction is in agreement with the observation that S. thermophilus transports lactose by exchange for galactose, a product of the lactose metabolism. The $V_{max}$ of 6000 nmol/mg × min reflects a turnover of ~7 s$^{-1}$, when it is assumed that all the molecules are reconstituted functionally. With an expression level of LacS in wild type S. thermophilus of 1–2% of total cell protein, the turnover number of 7 s$^{-1}$ reflects an uptake rate of 60–120 nmol of lactose/min × mg of total cell protein, which is similar to the in vivo lactose utilization (glycolysis) rate.

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