Amplified expression, purification and functional reconstitution of the dipeptide and tripeptide transport protein of *Lactococcus lactis*

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Transport of hydrophilic dipeptides and tripeptides into *Lactococcus lactis* is mediated by a proton-motive-force-driven peptide-transport protein (DtpT) that shares similarity to eukaryotic peptide transporters, e.g. from yeasts, plants, and the kidney and small intestine of rabbit, man and rat. The expression level of DtpT protein in *L. lactis* was increased (20–40-fold) to approximately 10% of total integral membrane protein by means of a low-copy-number vector and selecting the appropriate growth conditions. Membrane vesicles bearing the DtpT-His<sub>6</sub> protein (containing a C-terminal factor-Xa cleavage site and a six-histidine-tag) showed a Pro-Ala uptake activity that was half that of membranes containing the wild-type protein. The activity in the DtpT-His<sub>6</sub> membrane vesicles increased at least 50% upon removal of the His<sub>6</sub> tag from the protein. More than 95% DtpT was solubilized from *L. lactis* membranes in the presence of 1% (mass/vol.) n-dodecyl-β-D-maltoside, and approximately 2 mg DtpT-His<sub>6</sub>, was purified by Ni<sup>2+</sup>-chelate affinity chromatography from 100 mg membrane protein. Purified DtpT-His<sub>6</sub> was reconstituted unidirectionally into detergent-saturated formed liposomes, which were prepared from *Escherichia coli* phospholipid and egg phosphatidylcholine; the detergent was removed by adsorption to polystyrene beads. The highest uptake activities were obtained when DtpT was incorporated into liposomes that were treated with a low amount of n-dodecyl-β-D-maltoside (onset of liposome solubilization). The uptake activity could be improved by addition of NaCl (200 mM) and lipids (2 mg/ml) during the solubilization, purification and reconstitution steps.

*Keywords*: peptide transport; membrane reconstitution; proteoliposome; amplified expression.

The dipeptide and tripeptide transport protein (DtpT) of *Lactococcus lactis* encompasses a single polypeptide of 463 amino acid residues (Hagting et al., 1994). DtpT is similar to the eukaryotic proton-motive-force-dependent peptide-transport proteins of the PTR family (Steiner et al., 1995) and a peptide-transport protein of *Lactobacillus helveticus* (Nakajima et al., 1997). Examination of the hydropathy profile and analysis of its membrane topology, using the activities of DtpT-reporter fusion proteins and the pattern of labeling of single cysteine mutants by membrane-impermeable sulfhydryl reagents, suggests that the structure of DtpT corresponds to a bundle of 12 α helices with the N and C termini located intracellularly (Hagting et al., 1997).

To further study the structure/function relationships of DtpT, it was necessary to purify the protein and to develop methods for efficient reconstitution into artificial membranes. In these proteoliposomes, the ionic composition on both sides of the membrane is determined by the experimental design, and peptide transport is not influenced by hydrolysis of the substrate. The large-scale purification of (membrane) proteins is highly facilitated by overexpression of the polypeptide, for which E. coli is often the preferred host. However, the organism has been most successful for the amplification of endogenous membrane proteins (homologous expression); the sugar-transport proteins LacY, AraE, XylE, FucP, MeiB and GalP, the proline-transporter PutP, and the Na""/H"" antiporter NhaA have been expressed to levels exceeding 5% of total membrane protein (Poolman and Konings, 1993; Pourcher et al., 1995). The factors that determine the successful amplification of membrane proteins are largely unknown, but an obvious complication, compared with the overexpression of soluble proteins, relates to the proper membrane insertion and correct folding of the polypeptide when membranes are used as starting material for the isolation of the protein. When membrane insertion and subsequent folding are rate determining in the expression, it is not useful to increase transcription and/or translation. It has been observed that in some *E. coli* mutants, in which the rate of transcription is lower, several membrane proteins are successfully overproduced and inserted into the cytoplasmic membrane, while expression of these membrane proteins in wild-type *E. coli* is lethal (Miroux and Walker, 1996). However, many (heterologous) membrane proteins are toxic to *E. coli* cells, even when expressed at low levels. The deleterious effects probably arise from misfolded material that becomes associated with the cytoplasmic membrane. Overexpression can lead to the formation of inclusion bodies, which are generally less toxic or non-toxic, but denaturation and refolding of the protein are required prior to functional analysis.

The overexpression of membrane proteins in a homologous system may have some advantages over heterologous expression, even though very few studies have addressed this issue.
The expression of Lacs from *Streptococcus thermophilus* in *E. coli* is highest with the lacS promoter and the streptococcal Shine-Dalgarno sequences (~8% total membrane protein), which compares with about 2% total membrane protein when the tac or galP promoters of *E. coli* are used. The levels of Lacs can be increased to 25–30% total membrane protein, without noticeable growth inhibition and with high reproducibility, when the native expression signals are used in *S. thermophilus* (Knol et al., 1996). This study indicated that the expression level of the LacS protein in *E. coli* is highly influenced by the composition of the growth medium.

In the present paper, we report the amplified expression in *L. lactis*, purification and functional reconstitution of the dipeptide/tripeptide-transport protein DtpT. After optimization of the expression levels, choice of detergent and protein-stabilizing agents for solubilization, the His-tagged DtpT protein was purified in a single step by nickel-chelate affinity chromatography.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *E. coli* strains MC1061 (*Mla* [POZYA], araD139, *ara-leu*7697, *gallI*, *gallK*, *rspL*, *r,m*) and CJ236 (deriv, ung, thi, relA, *pCJ1105* (Cm*) were grown at 37°C, with vigorous aeration, in Luria broth, supplemented with carbencillin (50 μg/ml) when appropriate. *L. lactis* strains MG1363 and MG300 (MG1363, *ΔdtpT*) were grown at 28°C in M17 (Difco) or in chemically defined medium (medium A, Poolman and Konings, 1988), both at pH 6.4, and supplemented with 0.5% (mass/vol.) glucose plus 5 μg/ml erythromycin (when carrying plasmid vector).

**Synthesis of prolly-[14C]alacline.** Unlabeled alanine (11.8 μmol) was added to 10 ml 0.327 mM radiolabeled alanine (153 μCi/mmol), after which the mixture was freeze-dried and dissolved in 200 μl 150 mM NaOH. Equal amounts of 120 mM N,N'-dicyclohexylcarbodiimide and 300 mM N-butyloxycarbonylprolylalanine, dissolved in acetonitrile, were mixed and incubated for 3 h on ice to form the symmetric anhydride. The mixture was filtered over Whatman paper and the symmetric anhydride was added to the alanine solution (at a sevenfold molar excess over alanine), and incubated overnight at room temperature. The synthesis of N-butyloxycarbonylprolylalanine and the disappearance of alanine were verified by TLC, and the reaction mixture was concentrated overnight in a vacuum desiccator in the presence of silica gel to approximately 50 μl. To remove the N-butyloxycarbonyl group, 200 μl 3 M HCl was added, and the mixture was incubated for 1 h at room temperature. The disappearance of N-butyloxycarbonylprolylalanine and the appearance of Pro-Ala were verified by TLC, and the reaction mixture was concentrated overnight in a vacuum desiccator. The product was dissolved in 1.2 ml sterile water. The yield of the synthesis was about 60%, as determined from the radioactivity of the sample. The purity of the sample was determined by reverse-phase HPLC and TLC, and showed no detectable levels of alanine in the preparation.

**Molecular cloning.** Molecular-cloning techniques were performed essentially as described by Sambrook et al. (1989). Plasmids from *L. lactis* were isolated as described (Leenhouts et al., 1990). *L. lactis* was transformed by electroporation as described (Holo and Nes, 1989). All plasmids used in this study are listed in Table 1.

**Engineering of His, tag to wild-type DtpT.** Site-directed mutagenesis was used to generate a unique *MluI* restriction site that overlaps with the stop codon of the *dtpT* gene. The 3′ region of *dtpT* was isolated as a 1566-bp *KpnI*–*SalI* fragment from plasmid pDT3 and ligated into pSK(II)+. Single-stranded uracil-containing DNA of the resulting plasmid pSKF10 was isolated from *E. coli* CJ236 and served as a template for annealing of the oligonucleotide primer DtpTMlu (5′-GATGTTCTTACGC- GTAAAAGGATGA). The resulting plasmid (pSKF10Mlu) was transformed into *E. coli* MC1061, and the presence of the new *MluI* site was verified by restriction analysis. A linker, consisting of the two annealed oligonucleotides His-linS (5′-CGG- GATCTAGGCTGCATCGTATCCACCGATCTACTGTA) and His-linA (5′-CGCGCTGATGTGTTGATGATGACGA- CCGCACTCCTAA), that specify the factor-Xa cleavage site and a His, tag, were ligated into the *MluI* site of pSKF10Mlu. The insert and flanking regions were verified by nucleotide sequencing. The *KpnI* and SalI sites were used to isolate the 1603-bp fragment, which was exchanged for the corresponding fragment of pDT3, yielding pDT3HT. For expression of the His-tagged protein in *L. lactis*, the approximately 3570-bp *NcoI*-SalI fragment of pDT3HT was isolated and exchanged for the wild-type fragment of pGKF5, yielding pGKH.

**Transport assays.** Whole cells. Cells grown to an *A600* of 0.6 were harvested by centrifugation, washed twice, and suspended in 100 mM potassium phosphate, pH 6.5, to a final *A600* of approximately 25. The cells were de-energized with 10 mM 2-deoxyglucose for 20 min at 28°C. washed twice and suspended at approximately 0.5 mg/ml in 100 mM potassium phosphate, pH 6.5. After 3 min of energization with 25 mM glucose, Pro-[14C]Ala (40 μM) was added, and the uptake was assayed for different times. The reaction was stopped by diluting the mixture with 2 ml ice-cold 0.1 M LiCl, and the cells were collected on 0.45-μm cellulose acetate filters. The filters were washed with 2 ml LiCl. Transport assays were performed at 28°C.

Right-side-out membrane vesicles and proteoliposomes. Right-side-out membrane vesicles or proteoliposomes were fused with cytochrome-c-oxidase-containing liposomes by a freeze/thaw step and extrusion through 400-nm polycarbonate filters (Driessen et al., 1985; Mayer et al., 1986). The fused membranes were collected by centrifugation (280,000 × g, 15 min), and suspended at 1 mg protein/ml in 50 mM potassium phosphate, pH 6.5, 5 mM MgSO4. The concentrated membrane suspension was diluted 20-fold into oxygen-saturated 50 mM potassium phosphate, pH 6.5. 0.2 mM N,N,N′,N′-tetramethyl-p-phenylendiamine 20 μM cytochrome c. Pro-[14C]Ala (40 μM) was added 1 min after the addition of 10 mM ascorbate; the reaction mixture was gently stirred while water-saturated air was flushed over the solution. Further handling were the same as described for transport assays in whole cells, except that 0.45-μm cellulose nitrate filters were used.

Isolation of membranes. Right-side-out membrane vesicles of *L. lactis* were isolated as described (Otto et al., 1982), except that DNase and RNase were added to 100 μg/ml each. To remove peripheral membrane proteins and cytosolic contaminants, membrane vesicles were extracted with 5 M urea and 6% (mass/vol.) sodium cholate as described (Newman et al., 1981).

**Expression levels and raising of antibodies.** The amount of DtpT in the samples was estimated by immunodetection with antibodies raised against DtpT-His, in Freund’s adjuvant. The rabbit was boosted after 2 weeks with about 100 μg of the same preparation, and bled 8–12 days after each injection. The antibodies were used at 1:50,000 dilutions. For the western blot analyses, *L. lactis* cells were grown in M17 broth or medium A, supplemented with 0.5% glucose plus 5 μg/ml erythromycin, harvested in the late exponential phase of growth, washed once with water, and suspended in water to *A600* of 10. The cells were sonicated three times for 5 s on ice, and after addition of sample buffer, boiled for 10 min. The proteins (20 μg/lane) were separated by SDS/
Table 1. Plasmids used. Carb and Em indicate resistance to carbenicillin and erythromycin, respectively.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
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<tbody>
<tr>
<td>pSKII(+)</td>
<td>pBluescript II SK+, Carb, high-copy expression vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pGT120</td>
<td>Em, E. coli/L. lactis shuttle vector</td>
<td>Van der Vossen et al. (1985)</td>
</tr>
<tr>
<td>pGKV5</td>
<td>pGKV210, containing dtpT of L. lactis on a 4.2-kb EcoRI–SalI fragment</td>
<td>Hagting et al. (1994)</td>
</tr>
<tr>
<td>pTAQ1</td>
<td>pBR322, Carb, lacl behind penicillinase promoter, expression vector with tac promoter</td>
<td>Genencor Int.</td>
</tr>
<tr>
<td>pDT3</td>
<td>pTAQ1, carrying dtpT gene of L. lactis on a 3.5-kb HindIII–SalI fragment</td>
<td>Hagting et al. (1994)</td>
</tr>
<tr>
<td>pSKFl0</td>
<td>pSKII (+), carrying 3’ end of dtpT of L. lactis on a 1566-bp KpnI–SalI fragment</td>
<td>this work</td>
</tr>
<tr>
<td>pSKF10Mlu</td>
<td>pSKF10, with MluI site at the stop codon of dtpT</td>
<td>this work</td>
</tr>
<tr>
<td>pSKF10HT</td>
<td>pSKF10Mlu with linker coding for a factor-Xa cleavage site and a His, tag ligated in the MluI site</td>
<td>this work</td>
</tr>
<tr>
<td>pDT3HT</td>
<td>pDT3 with dtpT-H6 rather than dtpT</td>
<td>this work</td>
</tr>
<tr>
<td>pGKHT</td>
<td>pGKF5 with dtpT-H6 rather than dtpT</td>
<td>this work</td>
</tr>
<tr>
<td>pGKHT(T360C)</td>
<td>pGKF5 with dtpT-H6 rather than dtpT</td>
<td>this work</td>
</tr>
</tbody>
</table>

PAGE (12% polyacrylamide) and transferred to poly(vinylidene difluoride) (Millipore) sheets by semidry electroblotting (Kyhse-Andersen, 1984). Detection, by means of the Western-Light chemiluminescence-detection kit with CSPD as a substrate, was performed as recommended by the manufacturer (Tropix Inc.).

**Factor-Xa treatment.** To make the His, tag accessible for the protease from the inside and the outside of the membrane, right-side-out membrane vesicles (500 μg protein in 20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 2 mM CaCl2) were sonicated three times for 5 s on ice in the presence of 2.5 μg factor Xa. The mixture was incubated for 5 h at 23°C, and the membrane vesicles were collected by centrifugation (280000×g, 15 min), and suspended in 100 mM potassium phosphate, pH 6.5, 5 mM MgSO4.

**Solubilization.** Membrane vesicles (4 mg/ml) were solubilized in 50 mM potassium phosphate, pH 8.0, 400 mM NaCl, 10% (by vol.) glycerol, plus 1% (mass/vol.) n-dodecyl-β-D-maltoside (DodGlc), unless indicated otherwise in the legends to the figures. The mixture was incubated on ice for 20 min, and the insoluble material was removed by centrifugation (280000×g, 15 min).

**Purification of DtpT-His.** The solubilized membrane proteins were mixed with Ni2+-nitrilotriacetaete resin (about 0.5 mg DtpT-His/ml resin) equilibrated with 50 mM potassium phosphate, pH 8.0, 400 mM NaCl, 10% (by vol.) glycerol, 2 mg/ml *E. coli* lipids, and 0.1% (mass/vol.) DodGlc (buffer A), unless indicated otherwise. The mixture was incubated for 45 min at 4°C under continuous shaking, and subsequently poured into a column. The column was washed with 5 column vol. buffer A, followed by 5 column vol. buffer A supplemented with 15 mM imidazole. The protein was eluted with buffer A containing 100 mM imidazole and immediately used for reconstitution. All handlings were performed at 4°C.

**Reconstitution of DtpT-His, in proteoliposomes.** Reconstitution was performed essentially as described by Knol et al. (1996). A 3:1 mixture of purified *E. coli* total lipid extract (Kawaka et al., 1971) and egg yolk phosphatidylcholine was suspended in 50 mM potassium phosphate, pH 7.0, at 20 mg lipid/ml. Unilamellar vesicles with relatively homogenous size were made by dialysis of octylglucoside-dissolved lipids, followed by freezing in liquid nitrogen, slow thawing at room temperature, and extravision through a 0.4 nm polycarbonate filter. The liposomes were diluted to 4 mg phospholipid/ml and titrated with varying amounts of DodGlc, and incubated for 2 h at 20°C. The solubilization of the liposomes was followed by measuring the absorbance at 540 nm (Rigaud and Pitard, 1995). The detergent-treated liposomes were mixed with purified DtpT-His, at 100:1 (by mass) and incubated for 30 min at 20°C under gentle agitation, after which the detergent was removed by absorption to polystyrene beads (extensively washed with methanol and water; BioBeads SM-2, 80 mg/ml), for 2 h at 20°C, followed by 2 h and overnight at 4°C. For each period the polystyrene beads were replaced with new beads. The proteoliposomes were washed with 50 mM potassium phosphate, pH 7.0, concentrated by centrifugation, and stored in liquid nitrogen.

**Orientation of the reconstituted DtpT protein.** The mutant protein [Cys360]DtpT-His, was purified and reconstituted as described above. The proteoliposomes (0.1 mg/ml protein) were treated with 200 μM AMS or untreated for 30 min at 30°C. The membranes were washed twice (centrifugation for 15 min at 280000×g), suspended to 0.1 mg/ml protein in 50 mM potassium phosphate, pH 7.0, and incubated for 30 min at 30°C with 200 μM 3-(N-maleimidylpropionyl)biocytin (MPB). For the outside and inside labeling, the proteoliposome samples were sonicated with a microtip at an output of 4 μm (peak to peak) for 4 s in the presence of MPB. The 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.

**Miscellaneous.** Protein was assayed according to Lowry et al. (1951) with bovine serum albumin as a standard.

**Materials.** [14C]Alanine was obtained from Amersham, factor Xa from Biolabs, Ni2+-nitrilotriacetaete resin from Qiagen, Inc., BioBeads SM2 from Bio-Rad; n-octyl-β-D-glucopyranoside and Triton X-100 from Boehringer Mannheim; DodGlc from Sigma; and dodecyloctaglycol from Fluka Chemie AG. Total *E. coli* lipids and L-a-phosphatidylcholine from egg yolk were obtained from Avanti Polar lipids. All other materials were reagent grade and obtained from commercial sources.

**RESULTS**

**Overexpression in L. lactis.** For amplified expression, the dtpT gene and its promoter regions were placed in the *E. coli/L. lactis* shuttle vector pGKV210, and the resulting plasmid pGKF5 was used to transform *L. lactis* MG1363 and *L. lactis* AG300 (*dtpT*). The cells were grown in M17 broth or medium A. The initial rate of uptake of Pro-Ala in whole cells, at a substrate concentration of 40 μM, was 0.5 nmol · min⁻¹ · mg protein⁻¹ for MG1363/pGKV210 grown in M17 broth (Fig. 1A). The introduction of plasmid pGKF5 (multiple copies of *dtpT*) into this strain led to a ninefold increase of the initial uptake rate. A further 2.5-fold increase of the initial uptake rate was observed when the cells were grown in medium A. The transport activity was highest (20 nmol · min⁻¹ · mg protein⁻¹) when pGKF5 was introduced into MG1363, in which *dtpT* was present on the chromosome and the plasmid. To relate uptake activities to express-
Fig. 1. Effect of growth conditions and gene dosage on Pro-Ala transport (A) and the expression levels of DtpT (B, C) in *L. lactis*. (A) *L. lactis* strains AG300/pGKV210 (●), MG1363/pGKV210 (○), AG300/pGKF5 (■) and MG1363/pGKF5 (▲) were grown in M17 broth (open symbols) or medium A (closed symbols) both supplemented with 0.5% (mass/vol.) glucose and 5 μg/ml erythromycin. The transport assays in whole cells were performed as described in Materials and Methods at 40 μM Pro-[^14]CAla. The relative expression levels were estimated from densitometry of the immunoblots (B). Lanes 1–3, AG300/pGKV210, MG1363/pGKV210, AG300/pGKF5, respectively, grown in medium A supplemented with 0.5% (mass/vol.) glucose. The arrow indicates the position of DtpT.

Solubilization of DtpT. Different concentrations of octyl-[β-D-glucoside (1–4%), DodGlc₂ (0.2–1.5%), dodecyl octaglycol (0.5–2%) and Triton X-100 (0.5–2%) in 50 mM potassium phosphate, pH 8.0, 200 mM NaCl, 10% (by vol.) glycerol were tested for their ability to solubilize DtpT-His₉ from right-side-out membrane vesicles. The best results were obtained with 1% (mass/vol.) DodGlc₂, which solubilized more than 95% DtpT-His₉ (Fig. 3). All other detergents and other concentrations of DodGlc₂ tested resulted in a less efficient solubilization (<75% for octylglucoside, <60% for Triton X-100, <25% for dodecyl octaglycol) and/or aggregation of DtpT-His₉. Aggregation was observed on immunoblots as higher-order aggregates of DtpT-His₉, especially when high detergent concentrations were used (data not shown). The pH during solubilization was kept at 8.0, since the protein was subsequently used to bind to the Ni²⁺-nitrilotriacetate resin. Lowering of the pH to 7.5 or 7.0 resulted in a slightly less efficient solubilization with 1% (mass/vol.) DodGlc₂ as detergent. Solubilizations were also carried out with salt concentrations from 0 to 600 mM NaCl but the degree of solubilization was not much affected by the differences in ionic strength.
Purification and reconstitution. Solubilization of DtpT-His<sub>6</sub> from membranes of <i>L. lactis</i>/pGKF5 for 20 min with 1% (mass/vol.) DodGlc<sub>2</sub> in 50 mM potassium phosphate, pH 8.0, 200 mM NaCl, 10% (by vol.) glycerol was used in further studies, which involved purification of the protein by Ni<sup>2+</sup>-chelate affinity chromatography. Most contaminants were removed by washing of the column with 50 mM potassium phosphate, pH 8.0, 200 mM NaCl, 10% (by vol.) glycerol, 0.1% (mass/vol.) DodGlc<sub>2</sub> and 15 mM imidazole. The protein was eluted by raising the imidazole concentration to 100 mM. Samples of solubilized membrane vesicles and purified protein were analyzed by SDS/PAGE (Fig. 3) and showed that DtpT-His<sub>6</sub> could be isolated with a purity of greater than 90%. The yield of the procedure was typically 200 μg DtpT-His<sub>6</sub>, protein from 10 mg crude membrane vesicles. The purified protein was used to raise antibodies in rabbit.

DtpT-His<sub>6</sub> was immediately reconstituted after purification by mixing the purified protein with liposomes prepared from <i>E. coli</i> total lipid extract and egg yolk phosphatidylcholine at a ratio of 3:1 (by mass), 200 mM NaCl. The components were present during the solubilization and purification of DtpT. The uptake assay was performed with 40 μM Pro-[^14]C]Ala. Pro-[^14]C]Ala uptake was driven by a proton-motive force that was generated by the oxidation of ascorbate/N,N,N',N'-tetramethyl-p-phenylenediamin/cytochrome c via cytochrome-c oxidase. The variations in the initial rates were maximally ±30% when data from independent experiments were compared, which means that the effect of Pro-Ala is not significant. The standard buffer used for purification and reconstitution was: 50 mM potassium phosphate, pH 8.0, 200 mM NaCl, 10% (by vol.) glycerol and 0.1% (mass/vol.) DodGlc<sub>2</sub>.

Table 2. Effect of substrate, lipids and salt on the activity of reconstituted DtpT-His<sub>6</sub>

<table>
<thead>
<tr>
<th>Addition</th>
<th>Initial rate of uptake (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.8</td>
</tr>
<tr>
<td>Pro-Ala</td>
<td>1.2</td>
</tr>
<tr>
<td>Lipids</td>
<td>1.9</td>
</tr>
<tr>
<td>Pro-Ala plus lipids</td>
<td>2.1</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.8</td>
</tr>
<tr>
<td>Pro-Ala, lipids and NaCl</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Fig. 2. Activity of DtpT-His<sub>6</sub>. Pro-Ala uptake was assayed at 25 μM Pro-[^14]C]Ala. The inset shows the relative activities of Pro-Ala uptake in right-side-out membrane vesicles prepared from AG300/pGKF5 (1 and 2) and AG300/pGKHT (3 and 4). The right-side-out membrane vesicles were incubated for 5 h at 23°C without (-) or with (+) factor Xa. The activity of right-side-out AG300/pGKF5 membrane vesicles incubated without factor Xa was taken as 100%; the standard deviations in the activity measurements were less than 10%.

Fig. 3. Purification of DtpT-His<sub>6</sub>, as analysed by SDS/PAGE stained with Coomassie Brilliant Blue. A 12% SDS/PAGE gel was loaded with the following samples: molecular-mass markers (lane 1); supernatant (15 min at 280000×g) after solubilization of the membrane vesicles with 1% (mass/vol.) DodGlc<sub>2</sub> (lane 2); pellet after solubilization (lane 3); wash of Ni<sup>2+</sup>-nitrilotriacetate column with buffer A plus 15 mM imidazole (lane 4); and elution with 100 mM imidazole (lane 5).

Table 2. Effect of substrate, lipids and salt on the activity of reconstituted DtpT-His<sub>6</sub>. The additions were: 1 mM Pro-Ala, 2 mg/ml lipids [E. coli total lipid extract and egg yolk phosphatidylcholine at a ratio of 3:1 (by mass)], 200 mM NaCl. The components were present during the solubilization and purification of DtpT. The uptake assay was performed with 40 μM Pro-[^14]C]Ala. Pro-[^14]C]Ala uptake was driven by a proton-motive force that was generated by the oxidation of ascorbate/N,N,N',N'-tetramethyl-p-phenylenediamine/cytochrome c via cytochrome-c oxidase. The variations in the initial rates were maximally ±30% when data from independent experiments were compared, which means that the effect of Pro-Ala is not significant. The standard buffer used for purification and reconstitution was: 50 mM potassium phosphate, pH 8.0, 200 mM NaCl, 10% (by vol.) glycerol and 0.1% (mass/vol.) DodGlc<sub>2</sub>.

Fig. 4. Pro-[^14]C]Ala uptake activity of membrane-reconstituted DtpT-His<sub>6</sub>. Purified DtpT-His<sub>6</sub> in buffer A plus 0.1% (mass/vol.) DodGlc<sub>2</sub> was reconstituted in liposomes that were equilibrated for 1 h at 20°C with 3.8 (●), 6.4 (■) and 10.8 mM of DodGlc<sub>2</sub> (▲). The state of the liposomes, followed by measurement of the absorbance at 540 nm, 1 h after addition of DodGlc<sub>2</sub> is shown in the inset; the arrows indicate the three states of solubilization that were used for the reconstitution.
The samples shown in lanes 4 and 5 were sonicated in the presence of MPB to obtain 'maximum' biotinylation of the protein. The samples shown in lanes 1 and 2 were subjected to SDS-PAGE, electroblotted, and visualized by streptavidin-alkaline phosphatase (anti-strep) or anti-DtpT polyclonal Ig; some contaminating proteins are visible upon overnight exposure of the film.

Rigaud et al., (1988). To follow the physical state of the liposomes, the absorbance at 540 nm was measured upon addition of different amounts of detergent (Fig. 4). At three stages of solubilization of liposomes, purified DtpT-His6 was added, i.e. at the onset of solubilization (liposomes saturated with detergent), halfway through the breakdown of the liposomes, and at the point that the liposomes are fully solubilized (micellar state). For DodGlc, these points correspond to 3.8, 6.4 and 10.8 mM, respectively (Fig. 4). Following equilibration of the lipid/detergent mixture with DtpT-His6, the detergent was removed by adsorption to polystyrene beads. The proteoliposomes were fused with cytochrome-c-containing liposomes to generate a proton motive force to drive peptide uptake for prolonged periods of time. The uptake of Pro-[14C]Ala by DtpT-His6 reconstituted at the three points in the DodGlc titration curve is shown in Fig. 4. The highest activity was obtained when liposomes were titrated with a low detergent concentration. Using liposomes that were partially desintegrated, the activity was reduced by about 35%, while the activity was reduced by about 90% when mixed micelles of lipids and detergent were used.

The uptake activities in the proteoliposomes were not only dependent on the physical state of the liposomes at the beginning of the reconstitution, but also on the NaCl, lipid and substrate concentrations used during the purification and reconstitution (Table 2). The presence of NaCl NaCl (400 mM) during the solubilization, purification and reconstitution increased the initial uptake rate twofold and threefold, respectively. Less important seemed to be the presence of 1 mM Pro-Ala (carrier substrate) or glycerol, which is frequently used to stabilize membrane proteins in their solubilized state (Maloney and Ambudkar, 1989).

**Orientation of reconstituted DtpT protein.** The orientation of DtpT-His6, in proteoliposomes was assessed by monitoring the accessibility of the single cysteine in [Cys360]DtpT-His6, for a membrane-impermeable sulfhydryl reagent. Previous studies on the membrane topology of the DtpT protein have shown that Cys360 is present in a loop region at the outer surface of the membrane (Hagting et al., 1997). If [Cys360]DtpT-His6 is reconstituted in an inside-in orientation, Cys360 should react with the membrane-impermeable sulfhydryl reagent AMS, which should protect the protein from subsequent labeling with MPB (Loo and Clarke, 1995). MPB is weakly membrane permeable and upon prolonged incubation and at relatively high concentrations it will react with cysteines that are facing the inner surface of the proteoliposomes. The experiments indicate that [Cys360]DtpT-His6 was labeled by MPB and the labeling could not be enhanced by sonication of the samples (Fig. 5). Treatment of the proteoliposomes with AMS completely blocked the MPB labeling (Fig. 5), which strongly suggests that the protein is reconstituted with the cytoplasmic surface facing inwards, i.e. the orientation is the same as in the intact cell. The bottom panel of Fig. 5 shows an immunoblot with an anti-DtpT Ig, which indicates that equal amounts of [Cys360]DtpT-His6 were present in each lane.

**DISCUSSION**

In this paper we describe the amplified expression, purification and functional reconstitution of the dipeptide/tripeptide-transport protein of *L. lactis*. The expression of the DtpT protein was increased by using its own expression signals (promoter and Shine-Dalgarno sequences) and exploiting a convenient low-copy *L. lactis/E. coli* shuttle vector. We show that engineering of an affinity tag to the protein and use of nickel-chelate affinity chromatography provide an efficient and simple means to separate the carrier protein from (most of) the other membrane proteins. Reconstitution of the purified DtpT protein into detergent-stabilized liposomes, was unidirectional and highly reproducible.

Cell death (lysis) is frequently observed upon overproduction of heterologous membrane proteins in *E. coli* (Grisshammer and Tate, 1995). Overexpression of DtpT in *E. coli* was in most cases lethal (unpublished results). However, by means of a homologous expression system the problems of cell death could be avoided and rather high levels of expression were obtained. This is probably due to a better tuning of the rate of transcription/translation to the rate of membrane insertion and folding, which leads to higher amounts of functional protein in the membrane. As observed for the ATP-driven peptide-transport systems (Opp and Dpp) of *L. lactis*, the expression driven by the endogenous promoter was dependent on the composition of the growth medium. For DtpT, medium A without peptides yielded the highest level of expression. A similar observation has been made for the oligopeptide-transport system Opp (Detmers, F. M., unpublished results), whereas the Dpp system is induced by dipeptides and tripeptides containing branched-chain amino acids (Foucaud et al., 1995).

For the reconstitution of transport proteins, *n*-octyl-β-D-glucopyranoside solubilization of membrane vesicles and detergent removal by dilution or dialysis is often used (Baron and Thompson, 1975). This procedure has in many cases been improved by the addition of phospholipids and osmolytes (often glycerol), which seem to stabilize the proteins when they are in the detergent-solubilized state (Newman and Wilson, 1980; Maloney and Ambudkar, 1989; Poolman and Konings, 1993). Although a number of proteins have been reconstituted successfully after solubilization with *n*-octyl-β-D-glucopyranoside, several other proteins are irreversibly inactivated by this detergent (Knol, J., unpublished results). Therefore, we have tested a number of detergents for their ability to solubilize and retain activity of the DtpT protein. With a heterologous expression system this may require other detergents and conditions then when the same protein is purified from its natural host (Grisshammer and Tate, 1995). Very few studies have been performed in which membranes of lactic acid bacteria were used as source to isolate or purify a particular membrane protein. In our studies, we fa-
voured DodGlc, for the solubilization, purification and reconstitution of DtpT. With the purpose of reconstituting DtpT unidirectionally (Kno! et al., 1996), liposomes were titrated with detergent, and the \( A_{450} \) was measured to follow the physical state of the liposomes. For DodGlc, the steady-state absorbance values are reached very slowly (>60 min), which makes it time consuming to follow the solubilization by stepwise addition of DodGlc. Different amounts of DodGlc were added to different portions of liposomes and the \( A_{450} \) was determined after 1 h of incubation at 20°C (Fig. 4). At this time-point, the system has not been equilibrated fully, but it offers a good diagnostic measure of the physical state of the liposomes. These conditions were studied most extensively for the reconstitution of DtpT-His, with other detergents, such as dodecyl-octaglycerol or Triton X-100, the steady-state-absorbance values were achieved rapidly (<2 min), but the reconstitution of DtpT in these liposomes resulted in lower uptake activities (data not shown).

The advantage of reconstituting membrane proteins into detergent destabilized liposomes might be that one has better control of the incorporation of the protein into the bilayer and that the protein is faced with lower detergent concentrations. It has been suggested that the insertion of a protein into liposomes leads to proteoliposomes with better unidirectional protein insertion than when proteoliposomes are formed by coalescence of mixed micelles containing lipid, protein and detergent (Eytan, 1982; Helenius et al., 1981). A possible explanation for the unidirectional reconstitution of proteins into liposomes may be found in the difference in hydrophobicity between the outer and inner surfaces of the protein (Rigaud and Pitard, 1995). For secondary transport proteins the cytoplasmic loops that connect the transmembrane-spanning segments are usually much longer than the outer loops (Poolman and Konings, 1993), and therefore the cytoplasmic surface can be more hydrophilic, which would favour an inside-out reconstitution, as was observed for the lactose-transport protein (LacS) of *S. thermophilus* (Kno! et al., 1996). For DtpT we observed the opposite, i.e. the protein was reconstituted in an inside-in orientation (Fig. 5), which suggests that factors other than surface hydrophilicity affect the directed insertion of the DtpT protein into detergent destabilized liposomes.

The highest uptake activities of DtpT were observed when reconstitution was performed with liposomes that were fully saturated with detergent but still in the lamellar state (onset of solubilization), which could relate to the unidirectional incorporation under these conditions assuming that the kinetic parameters of transport are different for the protein in the inside-out and inside-in orientations. Since, the transport assays were performed at a substrate concentration far below the \( \text{P}_{\text{in}} \) compared with \( \text{P}_{\text{out}} \), a higher uptake rate if part of the carriers were reconstituted inside-out. On the other hand, we cannot exclude that the amount of (functionally) reconstituted protein is different under the various conditions tested or that high concentrations of DodGlc inactivate DtpT-His.

The initial uptake rate of Pro-Ala by DtpT-His, in whole cells was 4 nmol·mg protein\(^{-1}\)·min\(^{-1}\) (Fig. 2). A decrease in specific activity was observed when right-side-out membrane vesicles, prepared from cells expressing the His\(_{6}\)-tagged DtpT, were used to study peptide transport. However, in whole cells Pro-Ala is transported down the concentration gradient, because the dipeptide is immediately hydrolyzed. The lower specific uptake activity in the membrane vesicles and proteoliposomes may have been caused by substrate inhibition at the inner surface and/or a lower proton-motive force (and internal pH). The engineering of a His\(_{6}\) tag at the C terminus of DtpT decreased the uptake of Pro-Ala in whole cells by about 50%. A similar phenomenon was observed for His\(_{6}\)-tagged Tn10-specified tetracycline efflux antipporter TetA (Aldemna et al., 1996). Removal of the His\(_{6}\) tag from the DtpT fusion protein led to an increase of the activity. However, the specific activity of purified reconstituted DtpT-His\(_{6}\), is sufficient to study the specificity and kinetics peptide transport in detail, and allows us to evaluate the properties of any mutant DtpT protein in an artificial membrane system.

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**REFERENCES**


