Unconventional structural features of glutamate transporters
Slotboom, Dirk Jan

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2001

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Chapter 3

Purification and reconstitution of the glutamate carrier GltT of the thermophilic bacterium *Bacillus stearothermophilus*


Summary

An affinity tag consisting of six adjacent histidine residues followed by an enterokinase cleavage site was genetically engineered at the N-terminus of the glutamate transport protein GltT of the thermophilic bacterium *Bacillus stearothermophilus*. Two different expression plasmids were constructed to express the fusion protein in *Escherichia coli*. With a high copy plasmid, in which transcription was under control of the lac promoter, highest expression levels of the His-tagged transporter were observed in *E. coli* strain DH5α grown on rich medium without induction by IPTG. When transcription was under control of the arabinose promoter in a low copy plasmid, similar expression levels were observed in several *E. coli* strains after induction of the expression by L-arabinose. The His-tagged GltT protein was shown to transport glutamate and could be purified in a single step by Ni²⁺-NTA affinity chromatography after solubilization of the cytoplasmic membranes with the detergent Triton X-100. For reconstitution into proteoliposomes, the purified protein was mixed with detergent-destabilized, preformed liposomes, composed of *E. coli* phospholipids, followed by removal of the detergent with polystyrene beads. Active reconstitution was realized with a wide range of Triton X-100 concentrations. Neither the presence of glycerol, phospholipids or substrates of the transporter were necessary during the purification and reconstitution procedure to keep the enzyme in an active state.

In *B. stearothermophilus*, GltT translocates glutamate in symport with protons or sodium ions. In membrane vesicles derived from *E. coli* cells expressing GltT the Na⁺-ion dependency is lost (Tolner, B., Ubbink-Kok, T., Poolman, B. and Konings, W.N. (1995) *Mol. Microbiol*. 18: 123-133) suggesting a role for the lipid environment in the cation specificity. In agreement with the last observation, glutamate transport catalyzed by purified GltT reconstituted in *E. coli* phospholipid is driven by an electrochemical gradient of H⁺ but not of Na⁺.
Introduction

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. High affinity Na\(^+\) and K\(^+\) dependent glutamate transporters in presynaptic nerve terminals and glial cells help to terminate the transmission by removing the neurotransmitter from the synaptic cleft and are essential to keep the extracellular concentration of glutamate below neurotoxic levels. Impaired glutamate uptake has been implicated with neurodegenerative diseases (104,112,115,155). Glutamate transporters from different mammalian sources have been cloned and sequenced and were found to belong to a family of homologous transporter proteins in which also glutamate transporters from prokaryotic origin are found, such as GltT from *Bacillus stearothermophilus* (chapter 1, (145)). Analysis of the amino acid sequences and the hydropathy profiles of the members of the glutamate transporter family revealed that the proteins form a unique structural class of membrane proteins (chapters 1 and 2, (94,145)). Detailed knowledge about the structure of the proteins, which is indispensable for the unravelling of the molecular transport mechanism is lacking. We chose the glutamate transporter GltT of the thermophilic bacterium *Bacillus stearothermophilus* as model protein for structural studies.

Translocation of glutamate by GltT in *Bacillus stearothermophilus* is electrogenic and coupled to translocation of protons and sodium ions and therefore is driven by the proton and sodium ion motive forces. Studies with membrane vesicles from *B. stearothermophilus* showed that the sodium ion dependency of GltT significantly increased at elevated temperatures (160). The cation selectivity apparently is temperature dependent. The *gltT* gene has been expressed *Escherichia coli* and studies with membrane vesicles derived from the recombinant strain revealed a surprising result. Expression of GltT in *E. coli* resulted in complete loss of the sodium ion dependency; glutamate transport was driven by the proton motive force only (160). The data suggests that, in addition to the amino acid sequence, the cation-specificity of a carrier may depend also on conformation. Subtle conformational differences of GltT induced by the lipid environment and/or temperature may select for proton or sodium ion as the symported cation. Implicitly, it would mean that the difference between a proton and sodium ion binding site on the carrier is extremely small.

To determine such subtle conformational changes, and, more generally, to analyze the structure of the transporter, purification of GltT is essential. Here, we report the purification and functional reconstitution of GltT in *E. coli* phospholipids. The protein was expressed in *E. coli*, purified in a single step by His-tag affinity chromatography (62) and reconstituted by the detergent titration technique (83,131). Specific precautions that have been reported for the successful purification and reconstitution of several transporters from mesophiles were not necessary in the case of GltT. This may reflect the higher thermostability of this membrane protein from a thermophilic organism.

Materials and Methods

**Materials**

Ni\(^{2+}\)-NTA resin was obtained from QIAGEN, L-[\(^{14}\)C]-glutamate from Amersham, UK, synthetic oligonucleotides were obtained from Eurosequence, Groningen, The Netherlands.

**Bacterial strains and growth conditions**

The following *Escherichia coli* strains were used: DH5\(\alpha\), JM101, ECOMUT2 and ECOMUT1. ECOMUT1 lacks the genes coding for the glutamate transporters GltP and GltS (160) whereas ECOMUT2 lacks the *gltP* gene only. Unless stated otherwise, the cells were grown at 37 °C in LB medium. For expression of GltT derivatives from pBlueScript II KS (Stratagene, La Jolla, USA) derived plasmids IPTG was used at a final concentration of 0.1 mM. For protein expression from pBAD24 derived plasmids (53), 0.1% L-arabinose was
added at an optical density of 0.5 at 660 nm and cells were harvested 1.5 hr after induction. The antibiotics ampicillin and kanamycin were included when appropriate at concentrations of 100 µg/ml and 50 µg/ml, respectively.

**Construction of the expression vectors**

All genetic engineering was done using the standard procedures described in Sambrook et al., 1989 (138). A *NcoI* restriction site (CCATGG) was introduced with the ATG bases coinciding with the start codon of the *lacZ* gene on phagemid pBlueScript II KS by site-directed mutagenesis using the Kunkel method yielding vector pKS-*NcoI*. The base sequence of the mutagenic primer and the other oligonucleotides used in this study are listed in Table 1. Transformants were screened by restriction analysis of the plasmids. Subsequently, the unique *ClaI* site in the multiple cloning site of pKS-*NcoI* was opened and the overhangs were filled in with Klenow polymerase. Ligation yields vector pKS-*NcoI*∆*ClaI*. Deletion of the *ClaI* site results in a frame shift making the downstream *lacZ* sequences out of frame and resulting in white colonies on plates containing 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The frame shift was restored by digesting pKS-*NcoI*∆*ClaI* with *NcoI* and *BamHI*, which removes the destroyed *ClaI* region, and inserting a DNA linker coding for a His-tag of the appropriate length. Successful insertion of the linker was concluded from blue colouring on X-Gal plates. The 5' end of the linker has an overhang compatible with a *NcoI* cleavage site but destroys the site after ligation while the 3' end of the linker contains a *BamHI* cleavage site. The linker codes for a sequence of six histidines followed by an enterokinase proteolytic cleavage site. The coding regions are in frame with the *lacZ* gene. In front of the *BamHI* site, the linker contains a *NcoI* site (CCATGG) with the ATG bases in the same reading frame as the *lacZ* start codon. The linker was made by mixing 5 mg of two complementary synthetic oligonucleotides for 5 min at 37 °C (Table 1). The resulting vector is termed pKS-his.

<table>
<thead>
<tr>
<th>Purpose</th>
<th>DNA sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Construction pKS-<em>NcoI</em></td>
<td>5'-AATCATGGCCATGCTGGTTTC-3' ^</td>
</tr>
<tr>
<td>His-tag linker (sense)</td>
<td>5'-CATGATCACTACATCACTACATACGATGCAGATGACAAAGCCATGGGG-3' ^#</td>
</tr>
<tr>
<td>His-tag linker (antisense)</td>
<td>5'-GATCCCCCATGGCTTTGATCGTACATCGTGATGTGATGGTATG-3' ^8</td>
</tr>
<tr>
<td>PCR gltT (forward)</td>
<td>5'-GAAAAGGGGCGATCCATGGGAAAAATGGATTA-3' ^6</td>
</tr>
<tr>
<td>PCR gltT (backward)</td>
<td>5'-AAGCTGTTCTTCTGATAGGACAGCT-3' ^9</td>
</tr>
<tr>
<td>PCR gltThis (forward)</td>
<td>5'-CGAGAGGATTCCATGATGCGATCCATACCATACATACG-3' ^3</td>
</tr>
</tbody>
</table>

*indicated in bold are ^ the base changes, # the six histidine codons, ^ the *NcoI* and ^ XbaI restriction sites

Plasmid pGBT102 containing the *gltT* gene has been described by Tolner et al., 1992a (158) and was used to amplify the gene by the Polymerase Chain Reaction (PCR) technique. Primers gltT forward and gltT backward, which were used to amplify the 1.29 Kb DNA fragment coding for GltT introduced a *NcoI* cleavage site with the ATG bases coinciding with the start codon of the gene and an *XbaI* site 25 nucleotides downstream of the stop codon, respectively (Table 1). The introduction of the *NcoI* cleavage site results in a mutation in the second codon of *gltT* (Table 2). The purified PCR fragment was restricted with the *NcoI* and *XbaI* restriction enzymes and ligated into pKS-his and pKS-*NcoI* digested with the same enzymes. The resultant vectors are termed pGltThis and pGltT, respectively. The inserts were sequenced using an Amersham automated sequencer.

An L-arabinose inducible expression vector for GltT was made by cloning the *gltT*-gene encoding the glutamate transporter GltT of *B. stearothermophilus* with an N-terminal His-tag from plasmid pGltThis in the pBAD24 vector (53). The sequence encoding the His-tagged GltT was amplified using PCR from plasmid pGltThis with the primers gltT backward and gltT (Table 1). The amplified fragment was restricted with EcoRI and *XbaI* and cloned into the pBAD24 vector, which was restricted with the same enzymes. The resulting plasmid was termed pGltThisIII.

**Measurement of expression levels**

Membrane vesicles (20 µg protein) were run on SDS-polyacrylamide gels followed by transfer to PVDF membranes (Boehringer, Germany). Monoclonal antibodies directed against a 6 His-tag (Dianova, Germany) were used to detect His-tagged proteins. Antibodies were visualized using the Western-light chemiluminescence detection kit (Tropix, MA, USA).

**Purification of GltThis**

Cells from a 6 L culture in LB medium were harvested at an OD<sub>660</sub> of about 0.7, washed once with 250 mL 50 mM K<sup>+</sup>-phosphate pH 8 and resuspended in 40 mL of the same buffer containing 1 mM MgSO<sub>4</sub> and a trace amount of deoxyribonuclease. Cells were broken by three passages through a French Press cell operated at 10000 psi at 4 °C. Unbroken cells and debris were removed by centrifugation at 10000 g for 10 min at 4 °C and the membranes were collected from the supernatant by centrifugation at 100000 g for 90 min at 4 °C. Membranes were washed once with 50 mL potassium phosphate pH 8, 1 M NaCl and resuspended in 50 mM potassium
36 Purification of GltT

phosphate pH 8 at a protein concentration of 10 mg/mL. (DC protein assay from Bio-rad (Hercules, CA)). The membrane suspension was stored in liquid nitrogen. Membranes (4 mg/mL) were solubilized in 50 mM potassium phosphate pH 8, 400 mM NaCl, 20% glycerol and 1% Triton X-100. The solution was left on ice for 1 h with intermittent agitation. Undissolved material was removed by centrifugation at 250000 g for 20 min at 4 °C. The supernatant was mixed with Ni²⁺-NTA resin (100 µL/10 mg of protein) equilibrated in 50 mM potassium phosphate pH 8, 300 mM NaCl, 10% glycerol, 0.1% Triton X-100, 10 mM imidazole and incubated for 1 h at 4 °C under continuous shaking and, subsequently, poured into a column. The column was washed with 10 volumes of equilibration buffer containing 40 mM imidazole. The protein was eluted with 2 column volumes of the same buffer containing 150 mM imidazole and immediately used for reconstitution.

Reconstitution of GltT in proteoliposomes

Reconstitution was performed essentially as described by Knol et al., 1996 (83). A 3:1 (w/w) mixture of E. coli total lipid extract and egg yolk phosphatidylcholine was resuspended in 50 mM potassium phosphate pH 7 at a final concentration of 20 mg lipid per mL. The suspension was frozen in liquid nitrogen, thawed slowly and extruded through 400 nm polycarbonate membranes (Avestin). The liposomes thus obtained were diluted sixfold in the same buffer and treated with increasing amounts of Triton X-100. The solubilization of the liposomes was followed by measuring the optical density at 540 nm (131). Liposomes treated with different amounts of the detergent were mixed with purified GltT at a ratio of 10 µg of protein per 5 mg of lipid. The mixture was left at room temperature for 30 min after which the detergent was removed by three successive extractions with polystyrene beads (BioBeads, 80 mg/mL). The first extraction was done at room temperature for 2 h, the second and third at 4 °C for 2 and 16 h, respectively. The beads were removed by filtration over glass wool and the proteoliposomes were recovered by centrifugation at 25000 g for 20 min. The proteoliposomes were resuspended in the appropriate buffer and stored in liquid nitrogen.

Transport assays

Right-side-out (RSO) membrane vesicles. Membrane vesicles of E. coli were prepared by the osmotic lysis procedure as described by Kaback (1971) (68). The membranes were resuspended in 50 mM potassium phosphate pH 6 at a protein concentration of 15 mg/mL and stored in aliquots in liquid nitrogen. The membrane vesicles were energized by the potassium ascorbate/phenazine methosulfate (PMS) electron donor system. The membranes were diluted to a concentration of 0.6 mg/mL in 50 mM potassium phosphate pH 6, 2 mM MgSO₄ and 10 mM potassium ascorbate. The experiments were performed in 100 µL at 30 °C under a constant flow of water saturated air. PMS was added at a concentration of 100 µM and the proton motive force was allowed to develop for 1 min, after which L-[¹⁴C]-glutamate was added to a final concentration of 1.9 µM. The uptake was stopped by adding a 20-fold volume of ice cold 0.1 M LiCl solution, followed by immediate filtration over cellulose nitrate filters (0.45 µm, pore size). The filters were washed once with 2 mL of 0.1 M LiCl and assayed for radioactivity.

Proteoliposomes. For counterflow experiments, proteoliposomes were loaded with 50 mM potassium phosphate pH 7, 5 mM potassium glutamate, 2 mM MgSO₄ by freezing, thawing and extrusion through polycarbonate filters (400 nm pore size). The proteoliposomes were collected by centrifugation and resuspended in the same buffer at a concentration of approximately 0.2 µg of protein per µL. Counterflow was initiated by diluting the proteoliposomes 75-fold in the same buffer without unlabelled glutamate but with 1.3 µM L-[¹⁴C]-glutamate. The uptake was stopped as described above.

For assays of L-glutamate uptake driven by artificial gradients, the proteoliposomes were washed twice with 20 mM morpholine-ethanosulfinic acid (Mes) pH 6, 100 mM potassium acetate and concentrated as described above. Proton motive force driven uptake was initiated by diluting the proteoliposomes 75-fold into 120 mM Mes, 100 mM methylglucamine, 0.7 µM valinomycin and 1.3 µM L-[¹⁴C]-glutamate prewarmed at 30 °C. Both a proton motive force and sodium ion motive force was created by dilution into the same buffer containing 100 mM NaOH instead of methylglucamine. Control experiments were performed by diluting the proteoliposomes into the buffer with which they were loaded.

Results

Construction of the expression vectors pGltThis and pGltThisIII

A derivative of pBlueScript pKS II, named pK5-his, was constructed that facilitates the construction of genes fused downstream of a sequence coding for 6 histidines (His-tag) and an enterokinase cleavage site. In pKS-his the lac promoter, the ribosomal binding site and the
start codon of the lacZ gene are followed by in frame sequences coding for the His-tag and the enterokinase cleavage site and a NcoI restriction site (CCATGG) with the ATG bases in frame with the lacZ start codon. Downstream of the NcoI site are the BamHI to SacI restriction sites of the original pBlueScript multiple cloning site. The downstream lacZα sequence is in frame with the lacZ start codon which allows blue/white screening of inserts on X-Gal indicator plates.

The 1.3 kb gene coding for the glutamate carrier of Bacillus stearothermophilus, GltT (158), with the ATG bases of a NcoI cleavage site coinciding with the start codon was cloned in plasmid pKS-his yielding pGltThis. The NcoI site at the start of the gltT gene results in an Arg2Gly mutation in the protein. Under control of the lac promoter, pGltThis codes for GltT with an N-terminal His-tag and an enterokinase cleavage site.

The gltT gene was also cloned into vector pKS-NcoI, which results in the same construct except that the sequences coding for the His-tag and the enterokinase site are missing (construct pGltT). Plasmid pGltThisIII was constructed by cloning the sequence coding for His-tagged GltT from plasmid pGltThis in pBAD24 (53). pGltThisIII is a low copy expression vector in which GltThis is under control of the arabinose promoter.

The N-terminal amino acid sequences of the different constructs are indicated in Table 2. The inserts in the two plasmids were sequenced and found to be the same as the published base sequence of the gltT gene (158) except for the changes indicated in Table 2.

### Table 2. Amino acid sequences of the N-terminal part of the GltT constructs.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amino acid sequence$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GltT</td>
<td>MRKIG.....</td>
</tr>
<tr>
<td>GltT-NcoI</td>
<td>MGKIG.....</td>
</tr>
<tr>
<td>GltThis</td>
<td>MHHHHHHDDDDKAMGKIG.....</td>
</tr>
</tbody>
</table>

$^3$ the original GltT residues are indicated in bold and the enterokinase site is underlined.

#### Optimization of expression

The levels of expression of GltThis were measured in membrane vesicles prepared from E. coli strains, DH5α, JM101, ECOMUT1 and ECOMUT2, expressing the protein. Membrane vesicles were run on SDS polyacrylamide gels followed by Western blotting and immunodetection of proteins containing a His-tag using monoclonal antibodies against the His-tag. When GltThis was expressed from plasmid pGltThis in DH5α cells a His-tagged protein with an apparent molecular weight of 33 kDa was detected on the Western blot (Fig. 1). An apparent molecular weight of about 33 kDa is typical for an integral membrane protein of about 45 kDa and is in agreement with the observed molecular weight of GltT observed after $^{35}$S-methionine labelling (158). A second but much less intense band was visible at about twice the apparent molecular weight and corresponds most likely to the dimeric form of GltT. The levels of expression were remarkably different in the different strains. The highest expression was observed in DH5α, which is in line with the uptake activity observed in

![Dimer](image1)

Figure 1. Expression levels of GltThis. E. coli membranes were run on SDS polyacrylamide gels followed by Western blotting. His-tagged proteins were detected with antibodies derived against the His-tag. Lane 1: Membranes from E. coli strain DH5α expressing GltThis from plasmid pGltThis without induction by IPTG. Lanes 2–4: Membranes from E. coli strains DH5α, JM101 and ECOMUT2 expressing the protein from plasmid pGltThisIII after induction with L-arabinose.
membrane vesicles (below). Expression in strains JM101 and ECOMUT1 was significantly lower (not shown). Induction with IPTG resulted in higher levels of expression in strain JM101, which overproduces the repressor LacI. In ECOMUT1 and DH5α the level of expression was not significantly affected by IPTG. In these strains, the number of repressors relative to the high copy number of the plasmid is likely to be too low to cause a significant inhibition of transcription. Expression of GltThis from plasmid pGltThisIII, in which the arabinose promoter is used required the presence of L-arabinose in the growth medium. Similar expression levels were observed with this plasmid in DH5α, ECOMUT2 and JM101 when 0.1% L-arabinose was used to induce expression (Fig. 1). In this chapter strain DH5α harboring plasmid pGltThis grown in LB medium without IPTG was selected for purification of GltThis.

Activity of GltThis
Plasmids pGltThis and pGltT coding for the GltT transporter with and without the His-tag, respectively, were transformed to E. coli DH5α. Membrane vesicles with a right-side-out orientation were prepared of the recombinant strains and assayed for glutamate uptake activity. Since E. coli DH5α expresses its own glutamate carriers (28,159) membrane vesicles from DH5α transformed with plasmid pKS-his were used as a control. Fig. 2 shows that the initial rate of proton motive force driven uptake of glutamate in membrane vesicles derived from DH5α expressing GltThis is significantly higher than in the control vesicles indicating that the His-tagged glutamate carrier is actively expressed and that neither the presence of the His-tag nor the Arg2Gly mutation inactivates the transporter.

Glutamate uptake in vesicles derived from DH5α expressing GltT without His-tag gave similar results but the initial rate of uptake was significantly lower than observed with GltThis (not shown). This may be caused by a different specific activity of the mutant carriers, different levels of expression or a different energetic state of the membranes. The latter was clearly the case since uptake of proline catalyzed by the endogenous E. coli proline carrier in the two membrane preparations was significantly lower in the membranes expressing GltT without the His-tag. Moreover, DH5α cells expressing the latter transporter showed a strongly reduced growth rate, whereas the cells expressing the His-tagged protein showed only a moderately reduced growth rate. The strongly reduced growth rate and the decreased transport
activities suggest that overproduction of GltT has a negative effect on the metabolism most likely due to an impaired energy transduction. The His-tag may reduce the expression level resulting in well-coupled membranes vesicles. Since an antibody against GltT is not available to measure the level of expression of the protein a firm conclusion about the effect of the His-tag on the specific activity of the glutamate transporter is not possible.

Plasmid pGltThis was also transformed to *E. coli* ECOMUT1, a strain that lacks the endogenous secondary glutamate carriers (160). Expression of GltThis in ECOMUT1 resulted in low but significant uptake of glutamate in the membrane vesicles. Apparently, the level of expression in this strain is low.

**Purification of GltThis**

Membranes isolated by French Press treatment from *E. coli* DH5α expressing GltThis were solubilized by the detergent Triton X-100 in 50 mM potassium phosphate pH 8 containing 400 mM NaCl and 20% glycerol. The unsolubilized material was removed by centrifugation. The solubilized membranes were mixed with Ni^{2+}-NTA resin and incubated for 1 h in the cold room under continuous shaking. Subsequently, the mixture was poured into a column and washed with column buffer containing 40 mM imidazole. A small amount of GltThis eluted from the column in the wash step. The bulk of GltThis was eluted with buffer containing 150 mM imidazole. SDS-PAGE of the eluted fractions showed that the protein was essential pure as judged from both Coomassie Brilliant Blue (Fig. 3) and silver staining. The amount of GltThis in the membranes was not enough to see the protein after electrophoresis of the membrane fraction (Fig. 3). The yield of the procedure was typically 30 µg of pure protein from 4 mg of membrane protein which amounts to a fraction of about 0.7 % of total membrane protein (Table 3).

![Figure 3. Purification of GltThis](image)

Coomassie Brilliant Blue stained SDS-polyacrylamide gel of membrane vesicles derived from DH5α cells expressing GltThis (lane 1), the flow-through of the Ni^{2+}-NTA column (lane 2), the wash fraction of the Ni^{2+}-NTA column (lane 3) and 3 µg of purified GltThis (lane 4).

An amount of 10 µg of purified GltThis was incubated overnight at 23 C with 0.6 units of enterokinase in 20 mM Tris pH 7.4, 50 mM NaCl and 0.1 % Triton X100 in a total volume of 250 µL and loaded on a Ni^{2+}-NTA column. Essentially all the GltThis protein bound to the column and eluted with 150 mM of imidazole indicating that the His-tag was not removed by the proteolytic enzyme. Apparently, the enterokinase cleavage site is not accessible to enterokinase in the GltThis fusion protein.

**Reconstitution in proteoliposomes**

GltT was reconstituted by mixing the purified protein with preformed liposomes prepared from *E. coli* phospholipids at different stages of solubilization with Triton X-100. The solubilization by the detergent was followed by measuring light scattering in the sample spectrophotometrically (131). Titration of the liposomes with increasing concentrations of Triton X-100 results at first in an increase of light scattering caused by the incorporation of
detergent molecules in the liposomal bilayer until the liposomes are saturated with detergent (Fig. 4, inset). Increasing the concentration further gradually disintegrates the liposomes into mixed detergent/lipid micelles with a concomitant lowering of the light scattering until all liposomes are solubilized. An aliquot of freshly prepared purified GltTThis was added at three different points in the solubilization curve, (i) at the point of full saturation, (ii) halfway the breakdown of the liposomes and (iii) at the point where all liposomes are solubilized (see the arrows in Fig. 4). After incubation, the detergent was removed by polystyrene beads and the proteoliposomes recovered by centrifugation. Fig 4 shows glutamate counterflow activity in the three preparations. Clearly, the reconstituted purified GltTThis protein is active and the activity is, considering the complexity of the procedure, most likely not significantly different after the different reconstitution procedures.

Purification and reconstitution of other secondary transporters have been reported to require special precautions to preserve the activity of the protein (see Discussion). These include the presence of glycerol, phospholipids and substrates or combinations thereof during some or all steps of the solubilization, purification and reconstitution procedure. The procedure for GltTThis purification and reconstitution was repeated in the presence of the additions indicated in Table 3 and the resulting proteoliposomes were assayed for activity. The additions did not

Table 3. Effect of additions during solubilization, purification and reconstitution on the yield and activity of GltTThis.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Yield (µg/mg membrane protein)</th>
<th>Initial uptake rate(^a) (pmol/mg.min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>8.5</td>
<td>55.9</td>
</tr>
<tr>
<td>glycerol</td>
<td>6.5</td>
<td>83.9</td>
</tr>
<tr>
<td>glutamate</td>
<td>6.7</td>
<td>73.0</td>
</tr>
<tr>
<td>lipids</td>
<td>7.0</td>
<td>59.1</td>
</tr>
<tr>
<td>all</td>
<td>7.2</td>
<td>71.6</td>
</tr>
</tbody>
</table>

\(^a\) Concentrations during solubilization/purification, glycerol 10%, lipids 0.2 mg/mL and glutamate 1 mM. Concentrations during reconstitution, glycerol 2% and glutamate 0.2 mM. \(^b\) The uptake of glutamate was driven by an artificially imposed proton motive and ion motive force as described in the legend to Fig 5.
affect the purity of the preparation (not shown), and the differences in the yields were within experimental error. The specific initial uptake rates catalyzed by the reconstituted transporters under the different conditions are the same within the experimental error.

Cation specificity
Proteoliposomes of *E. coli* phospholipids containing purified GltT were prepared in the absence of Na⁺ ions. The proteoliposomes were loaded with 100 mM potassium acetate and diluted to a buffer without potassium ions and acetate in the presence of valinomycin. Passive diffusion of protonated acetic acid and valinomycin mediated potassium efflux down their concentration gradients result in the formation of a pH gradient, inside alkaline, and a membrane potential, inside negative. The resulting proton motive force drives the uptake of glutamate into the proteoliposomes (Fig. 5, ▼) showing that GltT does not require Na⁺ to translocate glutamate across the membrane. Dilution of the same proteoliposomes into 100 mM NaCl instead of methylglucamine results, in addition to the generated proton motive force, in an inward directed Na⁺ ion gradient. The additional sodium ion motive force has no effect on the initial rate of glutamate uptake nor on the level of accumulation (Fig. 5, ○). Similarly, uptake of glutamate driven by the membrane potential, in the absence of a pH gradient, was not affected by the generation of an additional sodium motive force (not shown). This data indicates that purified GltT reconstituted in *E. coli* phospholipid does not translocate Na⁺ ions which is consistent with the cation specificity of GltT in membrane vesicles derived from *E. coli* cells expressing GltT (160).

Discussion

The purification of membrane proteins requires that the proteins are taken out of their natural environment. At least for a while the phospholipid bilayer is replaced with the unnatural environment of the detergent micelle, which may destabilize the protein and result in unfolding. In many purification and reconstitution procedures of transport proteins special precautions have been described that were essential to keep the protein in the active state (125). For example, the lactose transporter of *E. coli*, LacY, could only be purified in a functional state when additional phospholipids were added in the detergent solubilized state (111). A high concentration of glycerol was essential in the purification of, for instance, the oxalate transporter of *Oxalobacter formigenes* (135). The presence of substrates of the transporter usually has a stabilizing effect as was claimed for the same carrier and also for the citrate carrier of *Klebsiella pneumoniae* (126). In this paper, the purification of the glutamate...
transporter of the thermophilic bacterium *B. stearothermophilus* is described. Proteins from thermophiles are likely to be more stable at ambient temperatures than proteins from mesophiles, a property that may prove to be of special importance in crystallization studies of membrane proteins. The properties of the GltT protein during the purification and reconstitution procedure support the claim of higher stability in that none of the above precautions is necessary to keep the protein in the functional state. In this respect it may be of interest that the alanine carrier of the thermophilic bacterium PS3 also could be purified and reconstituted in the absence of any special additions (60). It is not clear whether the presumed higher stability also relates to the ease by which the protein reconstitutes into liposomes. The stage of solubilization of the preformed liposomes by detergent is much less critical than has been observed for other transport proteins (131).

Studies in membrane vesicles of *B. stearothermophilus* have demonstrated that GltT catalyzes proton motive force driven glutamate uptake in the absence of Na$^+$ ions. In the presence of a Na$^+$ ion gradient the uptake activity was significantly higher, especially at elevated temperatures (160). Apparently, GltT translocates glutamate in symport with a proton or a Na$^+$ ion which places GltT in the same category as, for instance, the melibiose carrier MelB of *E. coli* (12). In contrast, a glutamate carrier of *E. coli*, GltS, a citrate carrier of *K. pneumoniae*, CitS, and all other amino acids transporters that have been studied in *B. stearothermophilus* translocate their substrates obligatory coupled to Na$^+$ ions (57,93,160). The differences between these two categories must reflect differences in the structure of the cation binding sites of the proteins. The cation binding pockets of the GltS and CitS type of carriers appears to be more stringent than those of the GltT and MelB type (96). The structural difference between binding pockets that accepts H$^+$ or Na$^+$ may be very small (see for instance (197-199)) and in the case of a pocket that accepts both H$^+$ and Na$^+$ a small change in conformation may result in a significant shift in the relative affinities for the two cations. This may be the explanation for the apparent loss of Na$^+$ dependency after expression of GltT in *E. coli* when the different lipid environment would cause a change in conformation of the cation binding pocket. The results presented here with the purified protein reconstituted in liposomes prepared from *E. coli* lipid are consistent with the observation made in membrane vesicles derived from *E. coli* cells expressing GltT, i.e. loss of Na$^+$ ion dependency. Reconstitution of purified GltT in liposomes prepared from lipids isolated from *B. stearothermophilus* will be required to confirm the influence of the lipid environment on cation selectivity.