Chapter 4

**Structural characterization of CitS, GltS and GltT transporters by single particle electron microscopy.**

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**ABSTRACT**

Three secondary transporter proteins: GltT of *Bacillus stearothermophilus*, CitS of *Klebsiella pneumoniae* and GltS of *Escherichia coli* were studied by single particle electron microscopy. The proteins were extracted from the membrane by detergent solubilization and purified to homogeneity by Ni$^{2+}$-affinity chromatography. Image processing showed a GltT particle with three-fold symmetry, which is in line with the trimer observed in the crystal structure of a homologous protein, GltPh of *Pyrococcus horikoshi*. The CitS protein showed up in two main views as a kidney-shaped particle and a biscuit-shaped particle, both with a long axis of 160 Å and a short axis of 84 Å. The GltS protein was similarly shaped but somewhat smaller with dimensions of 150 Å x 84 Å. Comparison of the shapes and dimensions of the CitS and GltS particles with the GltT particle suggested that the former two were in the dimeric state.

A 10 kDa protein tag (Biotin Acceptor Domain, BAD) was fused to the N-terminus of the CitS protein or inserted in the central cytoplasmic loop, which connects the two putative domains of the protein. Both constructs were shown to be active citrate transporters and in the dimeric state after purification in detergent. Single particle analysis of the two proteins revealed differently shaped particles relative to the untagged protein. N-terminally tagged CitS revealed a more globular shape relative to the wild type. The aspect ratios were 1.77 and 1.9, respectively. In contrast, the inserted BAD domain resulted in an extended particle with an aspect ratio of 2.14. A model is presented for the relative orientation of monomers in the CitS dimer.

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INTRODUCTION

Secondary transporters use the free energy stored in ion and/or solute gradients to drive the transport of a solute across the cytoplasmic or internal membranes of biological cells. They are widely spread throughout all kingdoms of life; they are found in every biological cell and a specific transporter protein can probably be found for every low-molecular weight compound in nature. Their high abundance is reflected in the great diversity of sequences coding for secondary transporters. The transporter classification system (TC system) developed in the Saier laboratory is based on sequence homology and lists some 95 different gene (super)families coding for secondary transporters (subclass TC 2.A) (Pao et al. 1999). Many of these families are likely to be evolutionary related, and the high number of encoded protein families is likely to represent a much smaller number of structures and an even smaller number of translocation mechanisms. Hydrophathy profile alignment has been proposed to group families of transport proteins or, more in general, membrane proteins with similar structures, into structural classes (Lolkema and Slotboom 1998). The hydrophathy profiles would report a specific folding pattern of proteins in membranes and, therefore, capable of detecting distant evolutionary relationships between protein families.

This study involved three different secondary transporters that are found in two structural classes. GltT of *Bacillus stearothermophilus* is a member of the Dicarboxylate/Amino Acid Cation Symporter (DAACS) family. GltT translocates glutamate and aspartate (Slotboom and Lolkema 1999) coupled to H^+. The DAACS family includes transporters that are found in neurons and glial cells in the mammalian central nervous system that catalyze the reuptake of the excitatory neurotransmitter glutamate from the synaptic cleft (Fairman et al. 1995; Kanai et al. 1992; Pines et al. 1992; and Storck et al. 1992). The DAACS family forms a structural class by itself, named Secondary Transporters [4] or (ST[4]). A member of the family (GltP_h of *Pyrococcus horikoshi*) has been crystallized and the three dimensional structure was solved at a resolution of 2.69 Å (Boudker and Gouaux 2007). The other two secondary transporters, CitS of *Klebsiella pneumoniae* and GltS of *Escherichia coli*, are both found in structural class ST[3]. CitS transports citrate in symport with two sodium ions and one proton. It belongs to the family of 2-hydroxycarboxylate transporters (2HCT) that contains transporters exclusively found in bacteria (Sobczak and
Lolkema 2003). GltS transports glutamate in symport with Na\(^+\) ions. GltS belongs to the Glutamate Sodium Symporter (ESS) family. Members of the 2HCT and ESS families cannot be regarded to be homologous by sequence similarity, but are classified in one and the same class because they show highly similar hydropathy profiles. Insight into the structure of class ST[3] proteins comes mainly from studies of CitS of *Klebsiella pneumon...* (van Geest and Lolkema 1999 and 2000; reviewed in Sobczak and Lolkema, 2005). The structural model of the transporters shows a core of two homologous domains consisting of five trans membrane segments (TMS) each that are connected by a large cytoplasmic loop region. Because of the odd number of helices, the two domains would have opposite orientations in the membrane (‘inverted topology’). In between the 4\(^{th}\) and 5\(^{th}\) TMS in each domain, the connecting loop folds back in between the TMSs to form so-called ‘pore loop’ or ‘reentrant loop’ structures. It is believed that in the 3D structure, the pore-loops of the two domains are in close vicinity and form the translocation pore. The CitS protein has an additional TMS at the N-terminal end of the core structure, placing the N-terminus in the cytoplasm. Alignment of the hydropathy profiles of the ESS and 2HCT families suggested that this additional TMS is missing in the GltS protein. Experimental evidence has been reported that confirms a similar membrane topology and the presence of the pore-loop structure in the C-terminal domain of the CitS and GltS proteins (Dobrowolski et al. 2008; Dobrowolski et al. 2009).

Our understanding of the structure of these proteins is still limited. A proven method to study the structure of (membrane) proteins is single particle analysis of electron microscopy (EM) projections. Over the last 25 years it has improved to a well-established technique to obtain information at a medium resolution (10–20 Å), and to near-atomic resolution for some highly symmetric objects (Frank et al. 2002; Van Heel et al. 2000). Single particle averaging is simple and attractive if applied to negatively stained specimens with a mass between about 100 and 2000 kDa, since one can process many thousands of projections within a few days, which is necessary to obtain high quality 2D projection maps. The statistical analysis and classification procedures used in single particle analysis are often effective in the sorting of different projection views originating from different conformations or subunit compositions.
In this study, the quaternary structure of the transporters GltT, CitS and GltS is studied using single particle electron microscopy. Since the particles are expected to be at the lower size limit of what is feasible for the technique (the monomeric molecular masses of GltT, CitS and GltS are 46.6, 49.1 and 43.5 kDa, respectively) the GltT protein was included as a reference particle. Ample evidence has been presented that the GltT protein assembles into a trimeric structure (Lolkema and Slotboom 1998; Boudker and Gouaux 2004; Groeneveld and Slotboom 2007). We present EM data that indicate that the other two transporters from the ESS and 2HCT families, share a similar subunit structure. Because of the small dimensions of proteins with a mass around 100 kDa, the low signal-to-noise ratio of transmission EM images of biomacromolecules, and the unstructured detergent shell around membrane proteins, single particle EM analysis is not (yet) able to reveal much detail in such proteins. We were able to show differences in size of dimers of CitS and GltS (~ 100 kDa) and in size and shape of CitS transporters to which the Biotin Acceptor Domain (BAD) of the oxaloacetate decarboxylase of *Klebsiella pneumoniae* with a mass of 10 kDa was attached.

**MATERIALS AND METHODS**

**Cell growth and membrane preparation**

*Escherichia coli* strain DH5α was used as a host to express the transporter proteins and derivatives. Freshly transformed bacteria were used to inoculate an over-night growing pre-culture. 25 ml of pre-culture was added to 1 liter of Luria Broth (LB) medium at 37°C and containing 50 µg/ml amphycilin (Roche Diagnostic GmbH, Manninheim, Germany) under continuous shaking (200 rpm). At an OD₆₆₀ of 0.6, 0.1% arabinose (Sigma-Aldrich GmbH, Steinheim, Germany) was added to induce expression of the transporters after which the culture was allowed to grow for an additional one hour. Subsequently cells were harvested by spinning at 8000 rpm in 4°C for 10 min, washed with 50 ml cold 50 mM KPi, pH 7, at 4°C and resuspended in 5 ml of the same buffer containing 1 µg/mL DNAse.

The cell suspension was passed three times through a pressure-cell-distrupter at 13,5 MPa at 4°C. Following a low spin at 8000 rpm for 10 min at 4°C to remove debris and unbroken cells, the supernatant was centrifuged at 80000 rpm for 25 min at 4°C (high spin). The membrane fraction was resuspended and washed once with 50 mM KPi, pH 7, buffer containing 1 M NaCl. The membranes were resuspended in 1 mL of 50 mM KPi, pH 7, buffer and stored in liquid N₂ until use.
**Purification of transporter proteins**

Transporter proteins were solubilized from the membranes by partial extraction. Membranes from a 1 L culture were incubated for 1 hour at 4°C under continuous shaking in 50 mM KPi, pH 7, buffer containing 400 mM NaCl, 10% glycerol and 0.5 % DDM. Undissolved membrane material which contained the transporter proteins was recovered by spinning at 80000 rpm for 25 min at 4°C. The extraction procedure was repeated with the same buffer with 1% Triton X-100 instead of DDM. The transporter proteins were recovered in the supernatant after spinning, passed through a 0.2 µm filter and applied to a 1mL bed volume Ni-NTA column (HisTrap HP) in an AKTA HPLC system. Before, the column was conditioned for 5 min at 1 ml/min flow rate of the carrier buffer (50 mM KPi, pH 8, 600 mM NaCl, 10% glycerol, 0.03% DDM, 20 mM imidazole). The loaded column was washed with 10 ml of 6% of the elution buffer (50 mM KPi pH 7, 600 mM NaCl, 10% glycerol, 0.03% DDM, 500 mM imidazole). The proteins were eluted in a linear gradient of 6% to 80% of the elution buffer. The OD$_{280}$ of the eluate wash measured continuously, after which the eluate was collected in fractions of 0.5 mL.

**Construction of plasmids**

Plasmids pBADGltS (Dobrowolski et al, 2005), pBADCitS (Sobczak and Lolkema, 2003), and pGltThis were constructed before (Slotboom et al, 1996), as well as pSB260 (van Geest and Lolkema, 1996). Plasmid pCitS-BAD1 was obtained by ligation of the gene coding for the BAD domain flanked at both sides by NcoI restriction sites in pSB260 into pBADCitS digested with the same restriction enzyme using standard procedures. Plasmid pCitS-BAD260 was obtained by recloning the BAD containing CitS gene in pSB260 into pBADCitS.

**Cross-linking with glutaraldehyde**

For each experiment, a 50 mM glutaraldehyde (Sigma-Aldrich GmbH, Steinheim, Germany) solution in water was freshly prepared. Protein samples were treated with 2 mM glutaraldehyde solution for 10 min at room temperature. The reaction was stopped by making the solution 100 mM in Tris-HCl pH 7 and incubating the sample for another 10 min at room temperature after which the samples were analyzed by SDS-PAGE.

**Right-side-out (RSO) membrane preparation** Cells cultures were grown, induced and harvested as described before. Cells of a 1 L culture were washed in 350 ml of 10 mM Tris-
HCl at 4°C and resuspended in 150 ml of 30 mM Tris-HCl, pH 8.0, 20 % sucrose at 37°C. After 10 min of incubation with 10 mM K-EDTA, pH 6, 100 µg/ml lysozyme was added and the cell suspension was incubated for an additional 15 min. Cells were collected by centrifugation, resuspended in 2 mL of 30 mM Tris-HCl pH 8.0, 20 % sucrose and, subsequently, diluted into 700 ml of KP, pH 7, at 37 °C. Following incubation for 15 min at the same temperature in the presence of 10 µg/ml DNAse and RNAse, 7 mL of a 1M MgSO₄ solution was added. After incubation for 15 min the membranes were collected by centrifugation for 60 min at 8000 rpm at 4 °C. The pellet was resuspended in 60 ml of 100 mM Kpi, pH 7, after which unbroken cells and debris was removed by spinning at 2500 rpm for 45 min at 4 °C (low spin). RSO membranes were collected from the supernatant by spinning at 20,000 rpm for 30 min at 4°C (high spin). RSO vesicles were resuspended in 50 mM KPi pH 7.0 and aliquoted in 150 µl portions that were stored at -80 °C. The concentration of the membrane protein in the RSO vesicles was assayed by the BioRad colorimetric protein assay.

**Transport studies**

RSO membrane vesicles were diluted to 1 mg/ml in 50 mM KPi pH 6 containing 100 mM NaCl. 100 µl aliquots were incubated for 2 min at 30°C with 10 mM K-ascorbate and 100 µM PMS under a flow of water-saturated air and continuous stirring with a magnetic bar. At t=0, 1 µl of 0.29 mM [1,5-¹⁴C]-citrate (114 mCi/mmol, Amersham Pharmacia, Roosendaal, The Netherlands) was added to final concentration of 2.9 µM. Uptake was stopped at 5, 10, 20, 40, 60 seconds by addition of 2 mL ice-cold 0.1 M LiCl followed by immediate filtering over cellulose nitrate filter (0.45 µm pore size) and washing of the filter with an additional 2 mL of the LiCl solution. Filters were collected and their radioactivity was measured in a liquid scintillation counter.

**SDS-PAGE**

Protein samples were mixed with loading buffer (250 mM Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 0.05% bromophenolblue) in a 1:4 ratio and loaded onto a polyacrylamide gel consisting of a stacking gel (4% polyacrylamide, 0.1% SDS, 0.375M Tris-HCl, pH 8.7) and separating gel (12% polyacrylamide, 0.1% SDS, 0.125M Tris-HCl, pH 6.8). The gel was run at a constant current of 25 mA for 45 min. The gel was stained in a buffer containing 25%
ETOH, 20% acetic acid, 0.25g/l Coomassie Brilliant blue R250, 0.25g/l CuSO₄ (Laemmli, 1970).

Electron Microscopy and Single-Particle Analysis
Negatively stained specimens of GltT, CitS and GltS were prepared with 2% uranyl acetate on glow-discharged carbon-coated copper grids. Electron microscopy was performed on a Philips CM12 electron microscope operated at 120 kV. Images were recorded with a Gatan 4K slow-scan CCD camera at 100,000 x magnification with a pixel size (after binning the images) of 3.00 Å at the specimen level, with “GRACE” software for semi-automated specimen selection and data acquisition. About 6,000 images were recorded and about 5,000 single particle projections from GltT, 80,000 projections from CitS, 30,000 from GltS and 28,000 single mutant N-terminus BAD CitS and CitS-BAD₂₆₀ projections were selected, respectively. Single-particle analysis was performed with the Groningen Image Processing (“GRIP”) software package on a PC cluster. Selected single-particle projections (96 x 96 pixel frame) were aligned by a multireference alignment and reference-free alignment procedures according to (Van Heel et al. 2000; Penczek et al. 1992). Next, particles were subjected to multivariate statistical analysis, followed by hierarchical ascendant classification (Van Heel et al. 2000). The resolution of the class averages was measured by Fourier Ring Correlation according to (Van Heel 1987). After several cycles of multireference alignments, statistical analysis and classification the best projections from each set were averaged.

RESULTS
Purification of GltS, CitS and GltT transporter proteins

Three secondary transporter proteins, GltT of Bacillus stearothermophilus, CitS of Klebsiella pneumoniae and GltS of Escherichia coli were expressed in E. coli DH5α harboring plasmids pBADGltT (Slotboom et al. 1999), pBADCitS (Sobczak and Lolkema 2003) and pBADGltS (Sobczak et al. 2007), respectively. The plasmids code for the transport proteins with N-terminal His₆-tags. The transporters were solubilized from the membrane by partial extraction using the detergents Triton X-100 and dodecylmaltoside as described in the Methods section. Solubilized proteins were loaded onto a Ni²⁺-NTA affinity column and, after washing, bound proteins were eluted from the column by a linear imidazole gradient ranging from 50 – 400 mM (Figure 1). Protein in the eluent was detected by the extinction at 280 nm
and the fractions were assayed for the presence of the transporter proteins by SDS-PAGE. The CitS and GltS proteins eluted at more or less the same imidazole concentration of 120 mM, while the GltT protein clearly bound stronger to the resin, eluting at 200 mM imidazole. SDS-PAGE following pooling of the peak fractions and concentration by ultrafiltration showed that the procedure resulted in highly pure protein preparations (Figure 1B). The apparent molecular masses of GltS, CitS and GltT were 35, 40 and 36 kDa. The yield of the procedure, which largely reflects the different levels of expression in the *Escherichia coli* host strain were 0.083, 0.3 and 0.21 mg of protein per liter of cell culture for GltS, CitS and GltT, respectively.

![Figure 1](image)

**Figure 1.** Ni$^{2+}$-affinity chromatography purification of GltS, CitS and GltT proteins. Main plot. Elution profile of GltS, CitS and GltT. The dashed line indicates the imidazole gradient. Insert. SDS-PAGE of purified GltS (lane 1), CitS (lane 2) and GltT (lane 3). Fractions of 1 mL collected at 26-29 mL of elution volume for CitS and GltS and 30-33 mL for GltT were pooled and concentrated by ultrafiltration before analyzing by SDS-PAGE. Estimated molecular masses were 35, 40 and 36 kDa, respectively. Left lane: marker proteins: 50 (a), 37 (b) and 25 (c) kDa. The gel was stained with Coomassie Brilliant Blue.
EM analysis of CitS, GltS and GltT.

Purified GltT, CitS and GltS transporter proteins were examined by electron microscopy (EM). Large numbers of monodisperse particles were present and a total number of about 130,000 projections of negatively stained GltT, CitS and GltS proteins was analyzed by single particle analysis. After several cycles of multi-reference alignment, multivariate statistical analysis and classification, final class sums from all analyzed particles (GltT, CitS and GltS) were obtained (Figure 2). The averaged EM projection maps of negatively stained GltT indicated high numbers of particles showing three-fold symmetry (Fig. 2A and B). The EM image of the GltT particle measures 118 Å in diameter and is likely to correspond to a top view projection of the GltT trimer (Yernool et al. 2004; Gendreau et al. 2004) which is in line with the known high resolution X-ray structure of the homologous transporter GltPh resolved previously (Boudker et al. 2007). GltPh shows a similar three fold symmetry when viewed along an axis perpendicular to the membrane (Fig. 1B). The space filling model, based on the x-ray structure of GltPh is a little bit smaller than the EM image of GltT because solubilized membrane proteins are surrounded by a detergent layer around the protein, which is not penetrated by the negative stain (Boekema et al. 1999). Particles with three-fold symmetry were almost the only type of projection that was observed. No side-views were found.

Analysis of 80,000 projections of the CitS protein indicated a high numbers of elongated particles (Fig. 2C and D). After several cycles of multireference alignments, statistical analysis and data classification, the best projections from each set were selected, combined and averaged. CitS appears to have two main types of projection views. One appears to be a biscuit-shape particle with a the long axis of 160 Å and a short axis of 84 Å (Fig. 2C). The second is a kidney-shape particle measuring 160 Å for the long axis and 81 Å for the short axis (Fig. 2D). CitS has been demonstrated to be a dimeric protein in detergent solution (Kastner et al, 2003; Pos et al, 1994; Veenhoff et al, 2001). Therefore, the biscuit-shaped particle is likely to reflect a top view because of its close-to 2-fold rotational symmetry, while the kidney-shaped projection is likely to represent a side view projection (mirror symmetry). The assignment will be discussed in more detail in the Discussion section.

A set of 30,000 EM projections of GltS were analyzed in a similar way as described above for GltT and CitS. The best projections from each set were averaged and the sums of the best classes are presented in Figure 2E and 2F. Similarly to CitS mainly two types of projection views of GltS were found (Figures 2E and 2F). The short axis of the projections were similar as observed for the CitS images (84 Å), while the long axis was significantly shorter (150 Å
The difference in shape between the two main projections of the GltS particle is smaller than observed for CitS (compare Figures 2C,D and 2D,F). The image shown in Figure 2D is somewhat asymmetric along the long axis suggesting a tilted particle. A small constriction in the middle of the projections may be observed suggesting similarity to the kidney-shaped projection of CitS in Figure 2D.

**Figure 2.** Single particle analysis of top-view projections of GltS, CitS and GltT. Averaged top view projection of GltT with a sum of 5,000 particles from *Bacillus stearothermophilus*. (A) High-resolution GltT<sub>Ph</sub> X-ray model in a position similar to the EM projection of image B. The mass around the X-ray model could suggest the extra stain or detergent layer around the protein. (C, D) Averaged top view projections of CitS with a sum of 80,000 particles from *Klebsiella pneumoniae*. (E, F) Averaged top view projections of GltS particles with a sum of 28,000 particles from *Escherichia coli*. Space bar is 100 nm.

**Biotin Acceptor Domain (BAD) tagged CitS proteins.**

The Biotin Acceptor Domain (BAD) domain of the oxaloacetate decarboxylase of *Klebsiella pneumoniae* is a ~10 kDa protein moiety. The gene coding for BAD was fused at the 5’ end of the *citS* gene yielding plasmid pCitS-BAD1 and inserted in the *citS* gene at the position corresponding to amino acid residue 260 of the CitS protein yielding plasmid pCitS-BAD260. The plasmids code for one hybrid protein consisting of the CitS with BAD fused at the N-terminus and the other with BAD inserted in the central cytoplasmic loop in between the two putative domains of CitS. Both constructs contain a His<sub>6</sub>-tag at the N-terminus.
Membrane vesicles with a right-side-out (RSO) orientation were prepared from *E. coli* DH5α cells expressing wild-type CitS and the two hybrid proteins and were assayed for citrate uptake activity. Membrane vesicles prepared from the host strain are completely devoid of citrate uptake activity because of the lack of expression of citrate transporters during aerobic growth of *E. coli* (not shown; Lolkema et al. 1994). The initial rate of uptake of citrate driven by a proton motive force imposed by the artificial electron donor system K-ascorbate/phenazine methosulfate (PMS) was roughly 40% less for the RSO membranes containing the two hybrid proteins than for those containing wild-type CitS (Figure 3A). The level of expression was determined by purifying the proteins from the membranes by Ni²⁺-NTA affinity chromatography using a batch-wise protocol. SDS-PAGE analysis of the partially purified proteins revealed that the two hybrid protein have apparent molecular masses of 50 kDa (Figure 3B, lanes 3 and 4) which is about 10 kDa more than the untagged CitS protein as expected (lane 2). A degradation product of CitS with an apparent molecular mass of 26 kDa was observed which is most likely the result of proteolytic activity during the lengthy procedure at elevated temperature of preparing RSO membranes. The intensity of the bands of the two hybrid proteins are more or less the same, but both were clearly less intense than observed for wild-type CitS (lane 2). The lower expression level of the two hybrid proteins largely explains the difference in citrate uptake activity of the different RSO membrane preparations in Figure 3A. Introduction of BAD at the N-terminus and at position 260 of the CitS protein does not seem to significantly affect the transport activity of the CitS moieties. The result is in line with the positive phenotype of *E. coli* cells expressing the hybrid proteins on Simmons agar indicator plates (van Geest and Lolkema 1999).

The CitS protein can be shown to be present as a dimer in the detergent solubilized state by cross-linking with glutaraldehyde. Treatment of CitS with 2 mM glutaraldehyde for 10 min shifts the protein band on SDS-PAGE up to a protein with an apparent molecular mass of 100 kDa (Figure 4). Pretreatment of the sample with SDS prevents the up shift demonstrating that cross-linking in the native state is not the result of collisions between monomeric particles. The two hybrid proteins were purified to homogeneity as described above. Similarly as observed for the CitS protein, treatment of the hybrid proteins with glutaraldehyde resulted in an upshift of the proteins on SDS-PAGE that was not observed with the denatured proteins (Figure 4). It follows that introduction of BAD at the N-terminus and at position 260 of the CitS protein does not interfere with the dimeric state of the CitS moieties. Treatment of the protein with glutaraldehyde results in more diffuse protein bands.
which is most likely the result of heterogeneity caused by random labelling of the protein and intramolecular cross-linking.

**Figure 3.** Transport activity of BAD-tagged CitS transporters in RSO membranes. (A) Uptake of $^{14}$C-citrate by RSO membranes containing wt-CitS line 1), N-terminal BAD-tagged CitS (CitS-BAD1, line 2) and CitS with BAD inserted at position 260 (CitS-BAD260, line 3). Membrane protein concentrations were 1 mg/ml. Citrate uptake was expressed in nmol per mg of membrane protein in the sample. (B) SDS PAGE of partial purified CitS moieties from the RSO membrane vesicles. Lane 1, wt-CitS, lane 2, CitS-BAD1, lane 3, CitS-BAD 260, left lane, marker proteins: 50 kDa (a), 37kDa (b), 25kDa(c).

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**Figure 4.** Cross-linking of wt-CitS, CitS-BAD1 and CitS-BAD260 by glutaraldehyde. The CitS moieties were treated or not with 2 mM glutaraldehyde in the absence and presence of 1 % SDS as indicated. Left lane, marker proteins, 50 kDa (a), 37 kDa (b), 25 kDa (c). Proteins were stained with Coomassie Brilliant Blue.
Electron Microscopy of BAD tagged CitS

The effect of the addition of the BAD domains on the sizes and shapes of the CitS protein was studied by EM. Analysis by multireference alignment, multivariate statistical analysis and classification of 14,000 projections indicated that both CitS-BAD1 and CitS-BAD260 yielded a single type of projection map, shown in Figures 5C and 5D, respectively. The general quality of the projection maps of the BAD tagged CitS proteins was lower than observed with wild-type CitS, resulting in a more blurred appearance. The poor quality of the images of both CitS with the BAD domain fused to the N-terminus and CitS with BAD inserted in cytoplasmic loop complicated the alignment and classification procedure which caused lower resolution. When compared to wild type CitS (Figure 5A and B) with a resolution of about 15 Å, the BAD tagged particles have a resolution of at best 20 Å.

In spite of the lower resolution, the sums obtained after reference alignment indicated changes in the overall shape of the BAD tagged CitS proteins. CitS with the BAD domain fused to the N-terminus resulted in a more globular shape of the particle (Fig. 5C). In contrast, CitS with BAD inserted in the cytoplasmic loop inbetween the two putative domains resulted in more extended particles (Fig. 5D). The dimensions of the different particles were summarized in Table 1. The BAD domain adds approximately 25% protein mass to the CitS particle. The increase of the surface of the EM projections was a bit higher with a factor of 1.3 and 1.37 for CitS-BAD1 and CitS-BAD260, respectively. This most likely due to the lower resolution of the latter. The aspect ratios of the particles were increasing in the order CitS-BAD1, CitS, CitS-BAD260.
Figure 5. A gallery of 2D projection maps from single particle EM of averaged BAD tagged CitS transporter proteins (A,B) top view of dimeric CitS. (C) Averaged projection of CitS with BAD inserted in the cytoplasmic loop of CitS (C) and with BAD fused to the N-terminus of CitS. (E,F) Schemes for BAD-tagged CitS proteins explaining the images in panels C and D. BAD domains (yellow), CitS (green). The thick solid line indicates the subunit interface, the thin line the putative interface between the N- and C-terminal domains of CitS. Space bar for all frames equals 100 nm.

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Table 1. Dimensions and aspect ratios of wt and BAD tagged CitS proteins. The predicted surface area was calculated assuming an increase in surface that is proportional to the increase in protein mass. Surface area of the wild-type is much smaller while the area of mutants is comparably similar. The dimension aspect ratios are consistently increasing in the order: CitS-BAD1, CitS and CitS-BAD260.
DISCUSSION

The investigation of the secondary transporters GltT, CitS and GltS by electron microscopy and single particle averaging gives several new insights into the architecture of these relatively small proteins. The glutamate transporter GltT found in *Bacillus stearothermophilus* and *Bacillus caldotenax* (Tolner et al. 1992) is a protein with a molecular mass of about 42 kDa. In the detergent solubilized state, the protein was shown to be a homotrimer by chemical cross-linking (Yernool et al. 2003) which is in line with the trimer observed in the high resolution structure of the homologous transporter Glt$_{ph}$ of the archaean *Pyrococcus horikoshi* (Yernool et al. 2004). The dominant projection image obtained after single particle averaging of purified GltT in detergent was a triangular particle with a clear three-fold rotational symmetry at about 14 Å resolution (Figure 2A). This strongly suggests that the image represents a view along an axis perpendicular to the membrane (top view). One of the reasons might be that the grids were glow discharged before preparing electron microscopy specimens. This enhances the absorption of particles with their hydrophilic parts, which results in predomination of top-view projections. A top view of a space filling model of the Glt$_{ph}$ trimeric structure (Figure 2B) superimposed on the image is a bit smaller which is due to the fact that solubilized membrane proteins are surrounded by a detergent layer, which is not penetrated by the negatively charged stain (Boekema et al. 1999). The EM analysis of GltT presented here, demonstrates that the shape of proteins as small as ~120kDa can be successfully determined by electron microscopy.

The citrate transporter CitS of *Klebsiella pneumoniae* is a protein of 46 kDa. No high resolution structure of the protein is available, but several lines of evidence indicate that CitS is a dimeric protein in detergent solution (Pos, Krupnik, see Sobczak and Lolkema 2003). EM analysis of purified CitS revealed two dominant classes of 2 D projection maps. Both projections showed elongated particles with a long axis about twice the length of the short axis (roughly 160 x 80 Å). One of the projections had a biscuit-shape, the other a kidney-shape (Figures 2C and D, respectively). The two classes represent two views of the protein rotated around the long axis. Secondary transporter proteins are largely embedded in the phospolipid bilayer without too much of their mass protruding into the inside or outside water phase. Considering that the thickness of the membrane layer is around 40-50 Å, the long axis
of both projections has to be in the plane of the membrane. Then, in the two projections, the short axis is either also in the plane of the membrane (view from within the membrane, side view) or perpendicular to the membrane (view on the membrane, top view). Since the orientation of an integral membrane protein in the membrane is fixed (positive-inside rule; von Heyne et al. 1991), a (homo)dimeric structure of a membrane protein forces certain symmetries upon the projections. In side view projection, one half should be the mirror image of the other half, which, in support for a dimeric structure of CitS, is observed for both projections. However, the top view should have two-fold rotational symmetry which is only compatible with the biscuit-shaped and not with the kidney-shaped image. It follows that the kidney-shaped image represents the side view of the CitS dimer. CitS would be a particle with an indentation at one side of the membrane at the interface of the subunits. The two EM images of the GltS protein reveal the same overall shape as observed for CitS but are somewhat smaller which is in line with their smaller molecular mass (Figure 2E,F and Table 1). The size of the particles suggests that GltS is a dimer as well. The shape is resembling the top view image of CitS while the corresponding side view was not observed. It is concluded that the dimeric structure of those two proteins is preserved despite their far evolutionary distance.

The structural model for the monomeric subunits of CitS and GltS consists of two homologous domains with the substrate and co-ion translocation sites at the interface of the two domains (Sobczak and Lolkema, 2005). In a simple three-dimensional model, the loop that connects the two domains would be at the ‘closed’ side of the monomer (the ‘back’) and the N- and C-terminal ends of the protein at the ‘open’ opposite side (the ‘head’). We have attempted to determine the relative orientation of the monomers in the CitS dimer by tagging the central loop and the N-terminus with an additional protein mass and, subsequently, locating the additional mass in the EM images. The biotin acceptor domain (BAD) of the oxaloacetate decarboxylase of Klebsiella pneumoniae that was used as tag is a 10 kDa protein which amounts to ~20% of the transporter protein mass. Both CitS-BAD1 (N-terminus) and CitS-BAD260 (central loop) had retained the Na+–citrate transport activity and dimeric structure. The increase in surface of the EM images of the two tagged CitS proteins was indeed observed, but slightly more than expected (Table 1). This is probably related to the lower resolution obtained for the images of the tagged proteins which may be caused by a relatively high degree of flexibility between tag and transporter protein.
estimated at 20 Å, may also have blurred the difference between top views and side views resulting in only one type of projection maps (Fig. 5C and D). Nevertheless, tagging the CitS protein at these two different sites results in a remarkable difference in shape of the particles. Relative to wild-type CitS, CitS with the BAD domain fused at the N-terminus appeared to have more globular shape with an aspect ratio of 1.77 vs. 1.91 for the untagged protein (Table 1). In contrast, CitS with BAD inserted in the cytoplasmic loop resulted in more extended particles with an aspect ratio of 2.14. The position of the additional cmass in both images suggest that the cytoplasmic loop between the two domains is placed at the end of the long axis and not involved in formation of the subunit interface, while the N-terminus is located at the interface in the dimer (Fig. 5E). It is concluded that the CitS dimer is ‘head-to-head’.

REFERENCES


