doi:10.1016/j.jmb.2007.06.067 J. Mol. Biol. (2007) 372, 565–570

JMB

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

COMMUNICATION

Rigidity of the Subunit Interfaces of the Trimeric Glutamate Transporter GltT During Translocation

Maarten Groeneveld and Dirk-Jan Slotboom*

Department of Biochemistry University of Groningen Groningen Biomolecular Science and Biotechnology Institute Nijenborgh 4, 9747 AG Groningen, The Netherlands

*Corresponding author

Glutamate transporters are trimeric membrane proteins in which each protomer contains a separate translocation path. To determine whether structural rearrangements take place at the subunit interfaces during transport, intersubunit disulfide bridges were introduced in the bacterial transporter GltT. None of the intersubunit cross-links, which had been designed across the entire interface, affected the glutamate transport activity, indicating that the subunit interfaces are rigid during turnover.

Keywords: membrane protein; glutamate transport; oligomeric state; transport mechanism; subunit interaction

Background

Glutamate transporters form a large group of membrane proteins that catalyze the uptake of glutamate into cells. The thermodynamically unfavorable concentrative glutamate uptake is driven by coupled transport of protons, sodium ions and/or potassium ions down their electrochemical gradients across the membrane. In mammalians the transporters are involved in clearance of the neurotransmitter glutamate from the synaptic cleft, and in prokaryotes the transporters are involved in the uptake of nutrients, such as glutamate and aspartate.[1] The crystal structure of the aspartate transporter Glt_{ph} from the archaeon *Pyrococcus horikoshii* has been solved.[2] The protein is a homotrimer, and each protomer contains a substrate translocation path. The trimeric oligomeric state is conserved in bacterial and mammalian glutamate transporters.[2–6] Much progress has been made in the identification of the structural determinants of the substrate and cation binding sites of Glt_{ph} and mammalian transporters by crystallographic and mutagenesis experiments.[7–9] But why these proteins are trimeric remains unclear. Here, we show by immobilizing the subunit interfaces in the bacterial glutamate transporter GltT that large structural rearrangements at the interfaces are not required for substrate translocation.

E-mail address of the corresponding author: d.j.slotboom@rug.nl

Cross-linking of the interfaces

In the crystal structure of the aspartate transporter Glt_{ph} each of the interfaces between the protomers consists of three contact regions (Figure 1): Transmembrane helix 2 of one protomer and helix 4a of its neighbor; helices 4b of two neighboring protomers; and helix 5 of one protomer and helices 4c and 5 of its neighbor. Pairs of residues (one on either side of the interface) were selected with the C^{β} atoms 4–6 Å apart in the structure of Glt_{ph}. The amino acids at the homologous positions in the glutamate transporter GltT from *Bacillus stearothermophilus* were mutated to cysteines to allow intersubunit disulfide bond formation. GltT was chosen for these experiments because its activity can be measured readily. GltT shares 36% identical residues with Glt_{ph} and is also trimeric.[6] Double cysteine residues were introduced in all three regions forming the interface: between helix 2 and 4a (I40C/V135C and K46C/K140C), helix 4b and 4b (F143C/T147C and G144C/T147C), helix 4c and 5 (G164C/A185C and G164C/Y188C) and between neighboring helices 5 (K171C/Q177C, K171C/Q180C, P174C/Q177C and V175C/F178C).

The double cysteine mutants and a cysteine-less control were expressed in *Escherichia coli*, as described.[10,11] Membrane vesicles with a right-side-out orientation were isolated and glutamate transport activity was measured, as described in the legend to Figure 2. These experiments were done under reducing conditions (10 mM DTT) in order to prevent disulfide bond formation and thus to be able to compare the activities of the double mutants with the cysteine-less mutant. The cysteine-less mutant

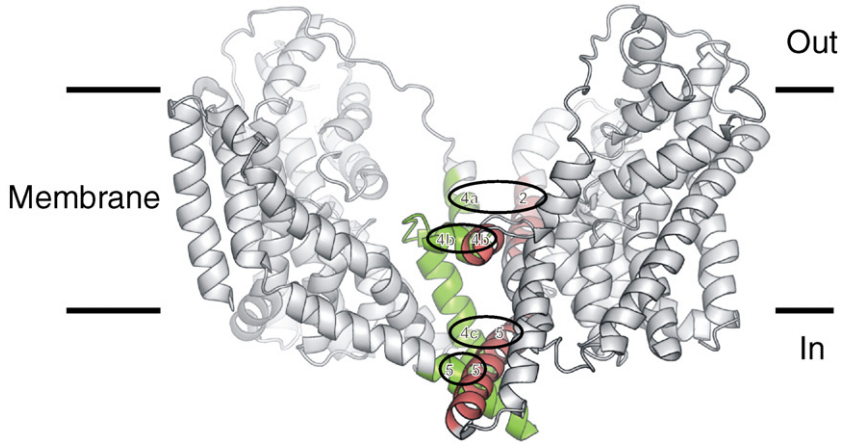

Figure 1. Structural model of Glt_{ph} (PBD accession code 1XFH) seen from the membrane plane. One of the three protomers was deleted to allow a clearer view of the interface between the two remaining protomers. The contact regions are colored red and green in either protomer. Numbering of the helices as by Yernool *et al.*[2] The structure was viewed in PyMOL [http://www.pymol.org].

displayed an initial uptake rate of 10.8 pmol s^{-1} mg^{-1} of membrane protein. Most double cysteine mutants had lower activities (Figure 2(a)), but the differences correlated well with the reduced expression levels of the mutants as estimated from Western blots (Figure 2(b)), indicating that the specific activities of the mutants were similar. When the expression levels were taken into account (as quantified from the Western blots), only the mutants I40C/V135C and K46C/K140C appeared to have lower specific activities than the cysteine-less mutant, but the differences were less than ~twofold. It must be noted though that quantification of the expression levels from the Western blots may not be very accurate, because the transfer of GltT from SDS–PAGE gels to the blot membranes was somewhat variable.

To determine whether the double cysteine mutants could form intersubunit disulfide bridges across the interfaces of the protomers, membrane vesicles were treated with either the oxidizing reagent copper phenantroline or the reducing agent DTT, to catalyze or prevent disulfide bond formation, respectively. Vesicles were run on SDS–PAGE gels, followed by Western blotting and detection of the His_6-tags, which were present at the N termini of all the mutants. Eight mutants, I40C/V135C, K46C/K140C, F143C/T147C, G144C/T147C, G164C/Y188C, K171C/Q177C K171C/Q180C and P174C/Q177C, were cross-linked readily by copper phenantroline and showed bands on the Western blots of the molecular mass of GltT trimers (Figure 3(a)). It is important to note that cross-linking was very efficient and that these mutants were completely converted into covalently cross-linked trimers with no residual monomers or dimers remaining. This shows that both cysteine residues in the double cysteine mutants must be involved in the cross-link formation. If one cysteine only had been involved in the cross-linking, monomers and dimers would be expected in non-reducing SDS–PAGE. For all mutants the optimal cross-linking conditions (copper phenantroline concentration, incubation time and temperature) were determined (shown in Figure 3(b) for I40C/V135C). Two mutants (V175C/F178C and G164C/A185C) could not be cross-linked under any of the conditions tested and these were taken along in the further analysis as negative controls. Two other mutants (K46C/K140C and G164C/Y188C) formed very stable cross-linked trimers that could be reduced only under conditions that are incompatible with activity assays (50 mM β-mercaptoethanol in denaturating loading buffer for SDS–PAGE gels). These mutants were excluded from the experiments described below.

To determine whether cross-linking of the protomer interfaces in the GltT trimer affected the transport activity, glutamate uptake was measured in membrane vesicles that had been treated either with copper phenantroline or with DTT. Oxidation with copper phenantroline was not expected to affect the activities of the cysteine-less mutant and the two double mutants that were used as negative controls (V175C/F178C and G164C/A185C). However, their initial transport rates were reduced by ~25% in the presence of the reagent (Figure 4). It is known that Cu^{2+} binds tightly to *E. coli* membrane vesicles,[12] and that the oxidizing conditions may affect their integrity, leading to slightly leaky vesicles. The shape of the uptake traces of the negative controls are consistent with this notion (Figure 4). Washing of the membrane vesicles with EDTA to remove excess copper ions partially reversed the effect, and treatment with DTT fully restored activity (not shown), but the latter was not compatible with maintaining disulfide cross-links in the double cysteine mutants. Therefore, we used the activity of the cysteine-less mutant as benchmark to determine the effect of intersubunit cross-linking on the transport activity of the mutants. The initial transport rates of all double cysteine mutants were affected to a similar extent by copper phenantroline treatment as the cysteine-less mutant (Figure 4). The ratios between the initial uptake rates in the oxidized and reduced vesicles were calculated for each mutant. *T*-tests showed that there were no significant differences between the ratio of any of the double cysteine mutants and that of the cysteine-less mutant.

Conclusions

Our work shows that it is very likely that the protomer interfaces of the glutamate transporter GltT