Inward-facing conformation of glutamate transporters as revealed by their inverted-topology structural repeats

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Glutamate transporters regulate synaptic concentrations of this neurotransmitter by coupling its flux to that of sodium and other cations. Available crystal structures of an archael homologue of these transporters, GltPh, resemble an extracellular-facing state, in which the bound substrate is occluded only by a small helical hairpin segment called HP2. However, a pathway to the cytoplasmic side of the membrane is not clearly apparent. We previously modeled an alternate state of a transporter from the neurotransmitter:sodium symporter family, which has an entirely different fold, solely on the presence of inverted-topology structural repeats. In GltPh, we identified two distinct sets of inverted-topology repeats and used these repeats to model an inward-facing conformation of the protein. To test this model, we introduced pairs of cysteines into the neuronal glutamate transporter EAAC1, at positions that are >27 Å apart in the crystal structures of GltPh, but ~10 Å apart in the inward-facing model. Transport by these mutants was activated by pretreatment with the reducing agent dithioretil. Subsequent treatment with the oxidizing agent copper(II)(1,10-phenantroline)3 abolished this activation.

In the present study, we show that inverted repeats in GltPh structures can be used to model an alternate conformation of EAATs in which the substrate binding site is exposed to the cytoplasm. The large conformational change predicted by this model is supported by conformation-dependent cross-linking of site-directed cysteine mutants of a mammalian glutamate transporter, EAAC1. The model is also in harmony with conformation-dependent accessibility of engineered cysteines to the membrane-permeant sulphydryl reagent N-ethyl maleimide (NEM) (12).
achieve the fit.

be rotated by

structure, which is important for the modeling. These segments have (SI) Table S1 and Fig. 2 TM8, albeit with reduced structural similarity [supporting information].

This relationship can be extended to include the entirety of TM7 and

ship between the two hairpin loops and half of their adjacent helices (6).

vious analysis of the structure of GltPh suggested a structural relation-

structural superposition highlights the shift in position of the hairpin-TM

position of the second half of each unit relative to the first half

segment I on segment II, we noticed a large difference in the

asymmetric structure of the whole protein (10). In GltPh, by

insertion in this region in human EAAT homologues.

Fig. 2. Structural similarity and differences between segments of GltPh. Structural alignments of (A) HP1 (yellow) plus TM7 (pale orange), against HP2 (orange) plus TM8 (magenta), and (B) TM 1 to 3 (shades of blue) with TM 4c to 6 (shades of green). In (C) we considered TM 1 to 3 (blue), plus HP2 and TM8 (red) as one pseudorepeat, and TM 4c-6 (green), HP1 and TM7 (orange) as a second repeat. Note that only the first three TMs were used for the fit: this structural superposition highlights the shift in position of the hairpin-TM motif with respect to the first three TMs of each pseudorepeat (arrow), which is a vertical curving movement (the approximate pivot point is shown as a circle). Figures were made using PyMOL (33).

We also investigated the six TM helices in the N-terminal half of GltPh, which comprise the so-called “cylinder.” This region has recently been suggested to contain a repeat of two TM helices (13). By including helix 4c (which spans the membrane), but excluding helices 4a and 4b (which do not span the membrane), we obtained a reasonable structural alignment between two 3-TM domains in this N-terminal cylinder (see Table S1 and Fig. 2B). The decision to exclude helices 4a and 4b (and the 3–4 loop) from the repeat is consistent with the large sequence insertion in this region in human EAAT homologues.

Based on the above analysis, the structure of GltPh can be described as containing four inverted-topology segments (which we refer to as I to IV) (Fig. 3A). That is, there is a pseudo 2-fold symmetry relationship between segments I and II, and another between segments III and IV.

In LeuT we observed a break in the symmetry between the five TM repeats, which was responsible for the formation of an asymmetric structure of the whole protein (10). In GltPh, by treating segments I and IV as one structural unit, and segments II and III as a second structural unit, and by superposing only segment I on segment II, we noticed a large difference in the position of the second half of each unit relative to the first half (Fig. 2C). Because the trimeric interface involving TMs 2, 4, and 5 is known to be unchanged during transport (14), we assume that any conformational changes within a given protomer would most likely involve segments III and IV rather than segments I and II. Consequently, it appears from Fig. 2C that the position of segment III is significantly lower within the membrane than the position of segment IV with respect to segments I or II.

Swapping the Conformations of Inverted Repeats Reveals a Cytoplasmic Facing Structure. We used the presence of inverted-topology repeats in GltPh to model an alternate conformation of GltPh using the approach used for the NSS proteins, namely swapping of the conformations of the repeats (10). Because there are four repeat segments in GltPh rather than two, as in LeuT, we adjusted the protocol slightly. Thus, the conformation of segment I was modeled using the structure of repeat II as a template, and vice versa, while the conformation of segment III was modeled on IV, and vice versa (Figs. S1A and S2 and Fig. 3B).

The model of GltPh generated by swapping the conformations of the repeats contains a large conformational change with respect to the crystal structure, and appears to represent the cytoplasm-facing state of the protein (Fig. S1 C and D and Fig. 4A). Because the helices forming the subunit interface in the trimer (particularly TMs 4a, 4b, 4c, and 5) are likely to be fixed, according to evidence from cysteine cross-linking (14), we discuss the changes in the rest of the protein with respect to these helices. The major difference that can be observed is that the
The core of the protein containing the binding site is predicted to have moved toward the cytoplasm by 15 to 25 Å relative to the subunit interface (see Fig. 4A and B). As a consequence, HP1 becomes exposed to the cytoplasmic solution, analogous to the extracellular exposure of HP2 in the x-ray structure (see Fig. S3 and Fig. 4A). This exposure of HP1 provides a means for release of substrate into the cytoplasm, namely by the flapping open of HP1, as has been suggested for HP2 in the extracellular conformation (7). In addition, the model predicts that HP2 will become buried under TMs 2 and 5 (see Fig. S3 and Fig. 4A), so that release of substrate and ions back to the extracellular environment would be inhibited significantly.

The model also suggests changes of 15 to 20 Å in the positions of TMs 7 and 8 to accompany the movements of HP1 and HP2 (see Fig. 4B). Such a concomitant movement may enable the binding site formed between these helices to be maintained. TMs 3 and 6, which shield TMs 7 and 8 from the lipid, are also shifted in the same direction, although to a lesser extent (10–15 Å). TM1 also moves somewhat, but in the opposite direction: that is, upwards in the membrane. In summary, the model predicts that the protein core consisting of HP1, TM7, HP2, and TM8 moves inward relative to the rest of the protein to form a cytoplasm-facing conformation that may correspond to an alternate state in the transport cycle.

**Cysteine Cross-Linking Supports Close Approach of TM2 and HP2.**

The large movements suggested by this model are supported by evidence for cysteine cross-linking in EAAT1 (15). Specifically, mutations to cysteine at positions equivalent to GltPh residues S52 or S55 (from TM2) and 364 (from HP2b) were found to cross-link in EAAT1, resulting in a loss of transporter activity (15). The Ca atoms of these residues are 27 to 29 Å apart in the x-ray structure of GltPh, and thus it is unlikely that they cross-link in this state (Fig. 5A). By contrast, in our model, TM2 has a significant interface with HP2. The distance between the Ca atoms in these cysteine pairs is ~10 Å, which is significantly closer to the 7 to 8 Å expected for disulfide formation (see Fig. 5B). To test whether the cross-linking of HP2b and TM2 has a higher probability under conditions in which the transporter becomes inward-facing, we analyzed the equivalent double-cysteine mutants of EAAC1 (also known as EAAT3), with cysteines introduced at V420 of HP2, and at either R61 or K64 of TM2.

Aspartate transport by either R61C/V420C or K64C/V420C was stimulated by 50 to 70% upon addition of the reducing agent dithiothreitol (DTT) (Fig. 6A and B), suggesting that the cysteine pairs spontaneously cross-link, similar to the observations for EAAT1 (15). Such cross-linking often inhibits transport (16–18), presumably because the disulfide imposes restrictions on the protein conformational changes necessary for transport, although such inhibition may also be the result of steric hindrance or another distortion introduced by the cross-link. Precipitation of 30 or 100 μM of the oxidizing agent copper (II)(1,10-phenanthroline), (CuPh) resulted only in modest inhibition of transport, supportive of the notion that many of the cysteine pairs are already cross-linked. An almost full inhibition of transport was observed when the CuPh concentration was increased to 300 μM (see Fig. 6A and B). Upon prior activation by DTT, the activity of the newly reduced transporters was fully sensitive to CuPh (see Fig. 6A and B).

These results indicate that when cross-linking is complete, all transporters are locked permanently in one conformation so that the translocation cycle is blocked. Thus, when transport is measured subsequent to exposure to lower concentrations of CuPh (such as 100 μM used in the experiment depicted in Fig. 7), the remaining activity is a read-out of the proportion of those transporters that were not yet cross-linked.

The effects of DTT and CuPh on transport were only observed for the double-cysteine mutants, and not for the equivalent single-cysteine mutants (V420C, R61C, or K64C) expressed either alone or together: for example, V420C with R61C or with K64C (Fig. 6C). These results suggest that both cysteines need to be present on the same polypeptide chain for cross-linking to
occur. Moreover, no effect of CuPh or DTT was seen with single-cysteine mutants in which the other residue was converted to either Ser or Ala, namely R61S/V420C, K64S/V420C, R61C/V420A, or K64C/V420A (see Fig. 6). Thus, it seems unlikely that inhibition occurs as a result of cross-linking of one of the introduced cysteines to an endogenous cysteine.

Cysteine Cross-Linking Can Be Modulated by Substrate and Inhibitors.

To determine the effect of the conformation of the transporter on the rate of cross-linking, the initial activation of the double-cysteine mutants by DTT was followed by incubation for 5 min with 100-μM CuPh under various conditions. Transport was subsequently measured after washing out the CuPh. When the sodium-containing medium was either supplemented with glutamate or replaced by potassium, conditions that promote the formation of the inward-facing conformation (see Fig. 1), the inhibition by 100-μM CuPh was potentiated (see Fig. 7). This suggests that the cysteine residues are indeed closer together in the inward-facing conformation. By contrast, addition of the nontransportable substrate analogue D,L-threo-benzyloxyaspartate (TBOA) reduced the cross-linking effect of CuPh (see Fig. 7), consistent with the expectation that TBOA increases the proportion of protein trapped in an outward-facing conformation in which the cysteines are far apart. In the absence of external sodium (replacement with choline) there was very little impact, if any, of glutamate or TBOA (Fig. S4), apparently because glutamate translocation and TBOA binding both require sodium (see Fig. 1).

In principle, the modulation of the inhibition by CuPh could be a result of changes in accessibility of the engineered cysteine residues, rather than in their distance. As a measure of their aqueous accessibility, we determined the effect of MTS reagents on transport by the single cysteine mutants, although it is true that these reagents are larger than the reactive oxygen species generated by CuPh. Preincubation of R61C or K64C with MTSES (2-sulphonoctoethyl-methanethiosulphonate) resulted in inhibition of transport, which was potentiated by either glutamate or external

**Fig. 6.** Effect of DTT and CuPh on d-[3H]aspartate transport by R61C/V420C and K64C/V420C. Transport of d-[3H]aspartate was measured in Xenopus laevis oocytes expressing the indicated mutants after pretreatment for 5 min in the presence or absence of 5-mM DTT, followed by a 5-min incubation with CuPh, as described in Materials and Methods. (A and B) Initial treatment in the presence or absence of 5-mM DTT was followed by a 5-min incubation with the indicated concentrations of CuPh. Results are expressed as percent of activity of oocytes that were preincubated twice for 5 min in frog Ringer’s solution without DTT or CuPh. (C) After preincubation with DTT, the oocytes were incubated for 5 min in the presence or absence of 100-μM CuPh. The results are expressed as the ratio of uptake in the presence of CuPh over that in its absence. “R61C co V420C” and “K64C co V420C” represent oocytes in which the two indicated cRNAs were coinjected.

**Fig. 7.** Effect of the medium composition during CuPh treatment on the transport activity of R61C/V420C and K64C/V420C. Oocytes expressing R61C/V420C (A) or K64C/V420C (B) were pretreated with 5-mM DTT for 5 min, followed by incubation for 5 min with 100-μM CuPh in either: frog Ringer’s solution in the absence or presence of either 1-mM l-glutamate or 60 μM of TBOA or frog Ringer’s solution in which all of the NaCl was replaced by KCl. The results are expressed as a percentage of activity of oocytes incubated without CuPh.
potassium (Fig. S5). However, TBOA, which protected against cross-linking of the cysteine pairs (see Fig. 7), did not modulate the inhibition of K61C or K64C by MTSES (see Fig. S5). For V420C, inhibition of transport by MTSET ([2-trimethylaminoethyl]methyl anethiosulfonate) was protected not only by TBOA but also by glutamate (see Fig. S5), which again is different from the cross-linking results. Thus, while the accessibility of the introduced cysteines to MTS reagents appears to be dependent on the conformational state of the transporter, the effects of substrates and substrate analogues on cross-linking cannot be explained merely in terms of such changes in accessibility.

Discussion

Experimental Evidence in Support of the Cytoplasm-Facing Model. Available x-ray crystal structures of GltPh bound to either the substrate aspartate, or to a nontransportable analogue TBOA, have similar conformations (6, 7). Both are essentially outward-facing, although in the absence of TBOA the helix-turn-helix segment HP2 provides a thin barrier that appears to flap open to expose the substrate binding site to the extracellular solution (see Fig. 4A) (13). To date, proposed mechanisms of the conformational change required to expose this aspartate binding site to the cytoplasmic solution have invoked relatively small movements in the protein, involving mainly HP1, with the rest of the structure remaining essentially unchanged (6, 7, 19). Our atomistic model suggests that the cytoplasm-facing conformation of GltPh is a symmetry-related version of the extracellular-facing structures, and that the inward- and outward-facing states interchange through a large, concerted movement of the core binding region of HP1, TM7, HP2, and TM8 (see Figs. 4 and 5).

The prediction of a large conformational change in the EAATs is supported by the observation that HP2 and TM2 can spontaneously cross-link in both EAAC1 (see Fig. 6) and EAAT1 (15), even though the equivalent residues are ∼27 Å apart in the x-ray structures (see Fig. 5A), much too far to allow formation of a disulfide bond. Our model predicts a marked reduction of this distance to ∼10 Å. Furthermore, the fact that oxidative cross-linking is potentiated under conditions with increased proportions of inward-facing transporters, and reduced when the fraction of outward-facing transporters is increased (see Figs. 1 and 7), supports the idea that HP2 and TM2 indeed become closer in the cytoplasm-facing conformation of the transporter. Large conformational changes are also supported by kinetic analysis of EAAC1, which showed that glutamate translocation is associated with energetic barriers of ∼100 kJ/mol (20). By contrast, distance measurements in EAAT3 using FRET suggest very little structural change in the protein (21). However, if the inward-facing states are relatively short-lived, then the latter measurements may not report on them, because FRET measures an average signal over all states.

Our model also predicts significant changes in solvent accessibility, so that most of HP1 and the cytoplasmic halves of TM7 and TM8 are accessible to intracellular solution in the model (Fig. S6 B and E), whereas they are buried within TM3, 5, 6, and 7 in the x-ray structure (Fig. S6 A and D). This prediction is consistent with the reactivity to NEM of cysteine residues introduced into these regions of a related transporter Glt1/EAAT2 (12); this reactivity increases in the presence of extracellular potassium. We were able to match calculated and measured accessibility of many, but not all, of the residues in this region for the model based on Asp-bound GltPh (see Fig. S6 B and E). Closer agreement, particularly for residues in HP1, was observed for a second model in which HP1 is more open (Fig. S6 C and F). Disagreement between measured and calculated accessibility at a few positions may reflect inaccuracies in the models, such as slight rotational or translational errors in helix position, which can affect the calculated accessibility of individual residues. Differences between GLT-1 and GltPh may also affect the comparison. Nevertheless, the overall trend in these data (i.e., that these segments become more accessible in inward-facing conformations) is very consistent with the proposed model (see Fig. S6).

Analogous cysteine-accessibility measurements for HP2 of GLT-1 revealed that substrates and inhibitors have complex effects on their reactivity to the cysteine modifier MTSET (22). Some residues, such as 448 and 449 (GLT-1 numbering), are protected from reacting with MTSET in the presence of glutamate, consistent with being buried under TM2 in the inward-facing model (see Fig. 4A), as well as with a likely reduction in mobility of HP2. However, several of the reactive residues in HP2 of GLT-1 are also protected by the nontransportable substrate analogue kainate (22), even though kainate likely holds the protein in an outward-facing conformation. This apparent contradiction can be explained by an increase in contacts between HP2 and TM4 (the loop between TM3 and 4) observed upon TBOA binding, which reduces the accessibility of residues in HP2 (7). Other residues in HP2 are, surprisingly, more accessible in the presence of TBOA than kainate (22).

Thus, the cysteine cross-linking, accessibility and other experimental data, are all consistent with the model of the inward-facing transporter. Clearly, however, ultimate proof of this prediction will require a crystal structure of a glutamate transporter trapped in this state.

A Mechanism of Alternating Access in EAATs. Based on our data, we propose a mechanism of alternating access in EAATs involving four major states of the transporter: two outward- and two inward-facing (Fig. S7). The first state is a substrate-free, outward-facing conformation (or ensemble thereof) in which HP2 is open, similar to TBOA-bound GltPh (7), as observed in molecular dynamics simulations (23, 24). Upon ion and substrate binding, HP2 would then close in to form an outward-facing, occluded state (see Fig. 4A).

In the next step, the conformation containing HP1, TM7, HP2, and TM8 would undergo the movement shown in Fig. 4 to form the inward-facing conformation of the model. This conformational change probably requires close packing of HP1 and HP2 against TMs 7 and 8, so that together they form a smooth sausage-shaped bundle. Thus, in acting as wedges that hold HP2 open, substrate analogues, such as TBOA, may inhibit their own transport by preventing this major conformational change. In the resultant inward-facing, occluded conformation, the thin intracellular gate would be formed by HP1. Release of substrates would then require opening of HP1 to form an inward-facing, open state, which has been modeled here based on the TBOA-bound structure (see Fig. S6F).

This four-state mechanism is consistent with trypsin cleavage of GLT-1, which suggests that glutamate is bound to two major conformations of the protein, only one of which can be inhibited by the nontransportable analogue dihydrokainate (25). Finally, binding of intracellular potassium would then favor closure of HP1, facilitating the reverse conformational change to the outward-facing state. A remarkable feature of the inward-facing model of GltPh is that it was constructed only based on the structures of the inverted-topology repeats inherent in the EAAT fold. An essential step in the modeling, therefore, was the identification of structural symmetry within the GltPh structure to cover as much of the structure as possible. In GltPh, one can think of segments I and IV as forming one single, noncontiguous pseudorepeat, while segments II and III form a second pseudorepeat with inverted topology to the first (see Fig. 3). We observed an asymmetry in those two pseudorepeats, so that segment III is
shifted vertically by ~10 Å compared to segment IV (see Fig. 2C). This asymmetry reflects the location of the core protein segment (III and IV) within the cylinder (I and II), so that swapping the conformations of the repeats creates a vertical displacement of the whole III-IV segment, resulting in an inward-facing state.

Such observations not only provide a technique for predicting alternate conformations of transporters, but also give further support for the appealing idea of a role for inverted-topology repeats in the transport mechanism (10, 13). In such a mechanism, each repeat has the ability to adopt two distinct conformations with similar energies (10). Interchange between those conformations thus allows the formation of two different, but symmetry-related structures with analogous substrate pathways leading to opposite sides of the membrane, thereby elegantly solving the main requirements for alternating access.

**Materials and Methods**

**Sequence Alignments.** An initial sequence alignment between the model and template was obtained from structure and ClustalW sequence alignments (26) of the four segments (see Fig. S1 and Fig. 3), and was adjusted to optimize, for example, binding site positions (see Fig. S2 and SI Materials and Methods).

**Construction of the Protomer Model.** Models of GlpPh protomers were built using Modeller 9v5 (27), where the template for each segment was the corresponding repeat from a GltPh crystal structure (PDB code 2NWL or 2NWW) (see Fig. 3). Distance constraints were added to keep the internal conformations of certain local regions similar to the x-ray structure (see SI Materials and Methods). These distance constraints did not affect the overall conformational change predicted by the model.

**Construction of the Trimer Model.** To construct trimeric models, we fitted the protomers onto the x-ray structure using helices TM3, 4a, 4b, TM4c, TM4, and TM6 (residues 82–253), and minimized changes at the protein-lipid interface (see SI Materials and Methods).

**Generation and Subcloning of Mutants.** The C-terminal histagged version of rabbit EAA1C (28, 29) in the vector pBluescript SK+ (Stratagene) was used as a parent for site-directed mutagenesis (30, 31). This was followed by subcloning of the mutants into its histagged EAA1C, residing in the oocyte expression vector pOG1 (Ambion Inc.) with PDB code 2NWL or 2NWW (see Fig. 3). Distance constraints were added to keep the internal conformations of certain local regions similar to the x-ray structure (see SI Materials and Methods). This structure agrees remarkably well with our prediction of the major conformational change that occurs during transport, providing convincing evidence that inverted-topology structural repeats are responsible for the creation of two symmetry-related states in transporter proteins. Furthermore, our data for EAA1C demonstrates that the predicted and observed conformational changes in the bacterial transporter also occur during transport of neuronal glutamate.

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Supporting Information

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SI Materials and Methods

Structural Alignments. Structural alignments were carried out between different fragments of the GltPh structure using the program SKA (1). To identify significant structural relationships, we only considered segments with a minimum of three secondary structure elements. It was assumed that the two helical hairpins, given their clear structural relationship, would constitute the basis of one repeat. Extending the segment containing HP2 as much as possible toward the C terminus, which optimizes the coverage of the structure, creates a unit consisting of HP2 and TM8. The two half-helices of TM7, when considered as one single helix, together with HP1, provide an analogous region that has the opposite topology. A relationship between TM7 and TM8 is supported by their structural alignment using TMalign (2) (Table S1). The structural superposition of HP1+TM7 and HP2+TM8 (Fig. 2A) with SKA has a protein structure distance score of ≈1.0, which is within the range observed for proteins classified as in the same “family” according to the Structural Classification of Proteins database (3).

N-terminal to the hairpins are six membrane-spanning helices and two nontransmembrane helices. The possible combinations of segments at least three secondary structure elements with inverted topologies are limited to TM2, TM3, and TM5. However, because TM4 contains three separate helices, we tested the effect of including the different helical segments of TM4. The most reasonable structural alignment was obtained when TM4c was considered to be equivalent to TM1 (see Table S1 and Fig. 2B). Helices 4a and 4b are therefore considered to be part of a long loop between the repeats, which is consistent with the long sequence insertion found in this region in human EAAT homologs. The main difference between TM3 and TM4 is that TM2 and 5 are curved in opposite directions.

Alignment of Repeat Sequences. To construct a model in which the conformations of the repeats were swapped, it was necessary to generate pairwise sequence alignments between segments I and II, and between segments III and IV. For the former, alignments were extracted from the structural alignment of those segments using SKA (1). That is, residues that were close to one another in space after the structural superposition were assumed to be aligned. For segments III and IV, we found that pairwise sequence alignments resulted in fewer gaps in the helices, and we thus used ClustalW (4) to align HP1 with HP2 (~16% identity) and TM7 with TM8 (~20% identity). The complete alignment was constructed according to Fig. 3 (Fig. S1A). A few gaps were manually removed from the alignments of TM2, 3, 5, and 6, so that these helices would be modeled as continuous helices, and a preliminary model of a protomer was constructed from this alignment (Fig. S1C).

Optimization of Alignment. As can be seen from Fig. S1C, the preliminary model demonstrates the same major conformational change described for the final model (Fig. S1D). Refinements were made to this model, to: (i) match the secondary structure observed the crystal structure with helical regions in the template as far as possible; (ii) reproduce the rotational (pitch) or translational (height) position of individual helices with respect to the membrane, aqueous solution or to other helices in the protein; and (iii) maintain the relative positions of binding site residues. These refinements create a more realistic model from physical-chemical and biological perspectives, and allow for a simpler comparison with the x-ray structure, while not affecting the major conformational change. They can be considered reasonable given the difficulty of accurate sequence and structural alignments at low sequence identities.

Specifically, the adjustments to the alignment were:

- The alignment of TM1, 2, and 3 was shifted by four residues (one helix turn) to increase the template coverage.
- The alignment of TM4c and TM5 was adjusted by four residues to maintain the contacts between protomers in the trimer, which are believed not to change during transport (5).
- These contacts are between TM2 and helix 4a (residue pairs L149:Y135 and K55:T140), between helices 4b of neighboring protomers (F143:A147 and G144:A147), between helix 4c and TM5 (A164:A193 and A164:K196), and between TMs 5 of neighboring protomers (S179:D185, S179:N188, T182:D185 and L183:A186).
- The alignment of the C-terminal half of TM6 was adjusted by one residue to remove an insertion in the TM domain.
- The alignment of HP1 was shifted by one to two residues, to remove a gap in HP1a and to place residues 276 to 279 at the tip of the hairpin (see Fig. S1 C and D).
- The binding-site residues 308 to 313 in TM7 were modeled without template and instead constraints were applied (see below).
- The alignment of HP2a was shifted by one residue to remove an insertion in the helix, while keeping residues 352 to 357 at its tip. An insertion was accommodated at the tip of this hairpin because the template contains fewer uncoiled residues.
- The alignment of the C-terminal half of TM8 was shifted by three residues to optimize the template coverage, while maintaining its helical pitch as in the crystal structure (e.g., with respect to TM7). Similarly, the alignment of the N-terminal half of TM8 was shifted by four residues. No template was used for residues 373 to 379 in the center of TM8, because the corresponding region is uncoiled, and instead constraints were applied (see below).

Importantly, none of these adjustments altered the overall shape of the model, and the essential features—namely the large shift of a domain containing HP1/TM7/HP2/TM8, as well as exposure of HP1 and burial of HP2—were conserved (see Fig. S1 C and D). This is because, in both models, the same TM helices are aligned to one another, while only the relative position of individual residues along these helices is changed.

The final alignment (Fig. S2) has a sequence identity of 9.8% (excluding 3L4).

Construction of Protomer Models. Models of GltPh protomers were built using the optimized sequence alignment (see Fig. S2), using the corresponding segment as a template. The loop between TMs 3 and 4 (3L4), including helices 4a and 4b (residues 111–149), which is not repeated, was kept the same as in PDB structure 2NWL. This was achieved by repositioning these residues in the template to the desired location of the loop in the final model. To further conserve the internal structure of the protomer (binding-site residues, orientation of individual helices, location of interface residues, and secondary structure), we added constraints for segments in which the template differed slightly from the x-ray structure. These constraints were taken from the x-ray structure using a cut-off distance of 6 Å. Specifically, we constrained:
• All residues in 3L4/TM4/TM5 (all atoms in residues 123–229), to fix the position of helices 4a and 4b within the interface (according to the constraints implied by ref. 5), and to improve the helicity of the cytoplasmic ends of TM4c and TM5;
• The binding-site residues in TM7 (between Cα atoms of residues 309–312);
• The internal structure of HP1 (between Cα atoms of the following residue pairs: 271 and 281, 268 and 284, 265 and 288, 275 and 281, as well as between Cα and Cβ atoms of residues 267–301);
• The relative orientation of the top of TM5 with respect to TM8 (between Cα atoms in residue 383 and in residues 208, 212, 216, and 220); and
• The helicity of the following regions: HP1a (residues 258–275), HP1b (residues 278–292), TM7 (residues 296–309 and 312–329), and TM5 (residues 386–401).

As can be seen from Fig. S1D, these changes serve to retain the internal structure of untemplated segments of the protomer, while not altering the relative movements of the repeats or the overall conformational change.

When using aspartate-bound GltPh as the template (PDB code 2NWL), 5,000 models were constructed, and the model with the lowest Modeller score was selected for further analysis. According to Procheck (6), four (1.2%) and three (0.9%) residues are in the generously allowed and disallowed regions, respectively, of the Ramachandran plot for this model. For a second model, built using the TBOA-bound structure of GltPh (PDB code 2NWW) as a template, the selected model from 2,000 attempts has equivalent Procheck values of five (1.4%) and four (1.2%), respectively.

Construction of Trimer Model. To construct trimeric models, we fitted the protomers onto the x-ray structure using helices TM3, 4a, 4b, TM4c, TM5, and TM6 (Cα atoms of residues 82–253). This maintained interfacial contacts in accordance with cross-linking experiments in GltT (5), and minimized changes at the protein-lipid interface. All atoms in the interface residues 137 to 147, as well as all side chains, were energy minimized with steepest descents for 2,000 steps using Charmm (7). The trimer models are deposited with identifiers PM0075966 (2NWL-based model) and PM0075968 (2NWW-based model) in the Protein Model Database (8) at http://mi.caspur.it/PMDB.

Transport Measurements. Uptake of D-[3H]aspartate was performed essentially as described (9), after pretreatment with DTT or CuPh as follows: for each condition, five oocytes were washed twice with 1 ml of frog Ringer’s solution containing 96-mM NaCl, 2-mM KCl, 1.8-mM CaCl2, 1-mM MgCl2, 5-mM Hepes, pH 7.5 and were subsequently incubated for 5 min with 1 ml of the same solution supplemented with 5 mM of DTT. Subsequently, the oocytes were washed twice with solutions of the compositions indicated in the figure legends, followed by a 5-min incubation of the oocytes with the same solution supplemented with 100 μM of CuPh. After washing the oocytes twice in frog Ringer’s solution, the oocytes were incubated for 20 min in 500 μl of the same solution supplemented with 1 μCi of D-[3H]aspartate (uptake was linear for at least 30 min). The oocytes were washed by passing through four wells filled with 4 ml frog Ringer’s solution followed by the individual incubation of each oocyte in 1% of SDS (500 μl/oocyte), followed by liquid scintillation counting. Each experiment was performed with two or three different batches of oocytes.

A. Preliminary Alignment

Fig. S1. Construction of the model of GltPh. (A) Preliminary sequence alignment between model and template sequences for internal-repeat homology modeling of GltPh. The alignment was constructed from the SKA structural alignment of segments I and II, and the ClustalW alignments of HP1 with HP2 and of TM7 with TM8, from which gaps in TMs 2, 3, 5, and 6 were removed (see SI Materials and Methods). Known TM helical segments according to PDB structure 2NWL are shown above and below the alignment for the model and template sequences, respectively. The helices are colored as in Fig. 3. A preliminary model of GltPh (C) was constructed using this alignment, and was compared to the x-ray structure (B) to guide adjustments of the alignment, which resulted in a refined model (D). For example, residues that are helical in the x-ray crystal structure were aligned wherever possible with residues that are helical in the corresponding part of the repeat [e.g., the cytoplasmic end of TM8 (magenta) is more helical in the final model than in the preliminary model]. The Cα atoms of pairs of specific residues from adjacent TM regions (shown as spheres of the same color) were used to help adjust the pitch/orientation of individual helices. For example, the alignment of residues in the tip of HP1 (red spheres) was incorrect in the preliminary model and was refined for the final model.
Fig. S2. Refined alignment between model and template sequences for internal-repeat homology model of GltPh. Coloring as shown in Fig. S1.
Fig. S3. The surface of the GltPh trimer in the extracellular-facing x-ray structure (Left) and the model of the cytoplasm-facing conformation (Right), viewed from extracellular (A) and cytoplasmic (B) sides of the membrane, with HP1 in yellow and HP2 in orange. The 3L4 loop and helix 4a are colored light green for reference. The tip of HP1 is exposed to the extracellular solution in the x-ray structure, but not in the model; analogously, the tip of HP2 is exposed to the cytoplasm only in the inward-facing model.
Fig. S4. Effect of CuPh treatment in the absence of sodium on the transport activity of R61C/V420C and K64C/V420C. After pretreatment of the oocytes expressing R61C/V420C (A) or K64C/V420C (B) with 5-mM DTT for 5 min, as described in SI Materials and Methods, the oocytes were washed with frog Ringer's solution in which all of the NaCl was replaced by ChCl. This was followed by incubation with 100-μM CuPh with the same solution in the absence or presence of either 1-mM L-glutamate or 60 μM of TBOA. Subsequently, sodium-dependent radioactive D-aspartate transport was measured, as described in SI Materials and Methods. The results are expressed as a percentage of activity of oocytes incubated without CuPh.
Fig. S5. Effect of the medium composition during treatment with MTS reagents on the transport activity of R61C, K64C and V420C. Oocytes expressing R61C (A), K64C (B), or V420C (C) were first washed with frog Ringer’s solution in the absence or presence of either 1-mM l-glutamate or 60-μM TBOA, or in frog Ringer’s solution in which all of the NaCl was replaced by KCl. The oocytes were then incubated for 5 min in solutions of the same compositions in the presence or absence of 10-mM MTSES (A and B) or 0.02-mM MTSET (C), followed by washing in frog Ringer’s solution, and then by transport measurements as described in SI Materials and Methods. The concentrations of MTSES and MTSET were chosen after preliminary titration experiments, to determine the concentration required for ~50% inhibition in sodium-containing medium for each mutant. This level of inhibition is optimal for determining the effect of the medium composition on the sensitivity of the cysteine mutants to sulfhydryl reagents. The results are expressed as the percentage of transport activity of oocytes incubated without MTS reagents.
Fig. S6. Increase in accessibility in HP1 (yellow), TM7 (wheat), and TM8 (pink) in the inward-facing model of GltPh is supported by cysteine accessibility measurements in GLT-1. (A) The reduction of glutamate uptake by NEM in single-cysteine mutants in the presence of potassium, as a percentage of uptake in the absence of NEM (in black) is a measure of the absolute accessibility in the inward-facing conformation (1). The calculated solvent accessible surface area of the equivalent residues of GltPh in an outward-facing conformation (PDB code 2NWL) is in blue. This accessibility was calculated as a percentage of the accessibility of the same amino acid type (X) in a reference GXG tripeptide (as described in ref. 2). (B and C) The increase in accessibility for inward-facing models of GltPh relative to the crystal structure (blue) is compared to the effect of extracellular potassium relative to sodium on the inhibition by NEM (black). The latter was calculated by subtracting the percentage inhibition by potassium from the inhibition by sodium. Models were built using either (B) an aspartate-bound structure (PDB code 2NWL), or (C) a TBOA-bound structure (PDB code 2NWW), as templates. In all plots, negative values have been set to zero. (D–F) Positions equivalent to residues that become significantly more accessible upon addition of potassium in GLT-1 (spheres) are shown in the x-ray structure (D), and in inward-facing models of GltPh built using a glutamate-bound structure (E) or a TBOA-bound structure (F).

Fig. S7. Proposed mechanism of glutamate/aspartate transport involving four major conformational states of the protein. TM 2 (blue), TM 5 (green), HP1 (yellow), and HP2 (orange) from one subunit are shown for clarity. Substrate is shown as a light blue triangle and ions are shown as colored spheres. The four states are: (i) outward-facing open; (ii and vi) outward-facing occluded; (iii and v) inward-facing occluded; and (iv) inward-facing open. Nontransportable analogues bind to GltPh to form a conformation similar to (i), and the aspartate/sodium-bound structures of GltPh correspond to (ii). Here, we propose atomistic models for states (iii) and (iv) and for the conformational change connecting (ii) and (iii).
<table>
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<tr>
<th>Name</th>
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<th>Name</th>
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</table>

Boldface indicates a structural alignment from which the sequence alignment was extracted to be used in the homology modeling. TM-scores of >0.4 indicate that the segments provide homology modeling templates defined as “Easy” [Zhang Y, Skolnick J (2004) Scoring function for automated assessment of protein structure template quality. Proteins 57:702–710.].

*Structure alignments calculated with TM-align instead of SKA, because they contained <3 secondary structure elements. Here, PSD is replaced by the TM-score.