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Transport Mechanism of Human Glutamate Transporter EAAT3

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UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

TRANSPORT MECHANISM OF HUMAN GLUTAMATE TRANSPORTER EAAT3

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Excitatory amino acid transporters (EAATs) remove glutamate from synapses in a reuptake process driven by the Na\(^+\) gradient to prevent glutamate concentrations from reaching neurotoxic levels. Malfunction of this reuptake mechanism is implicated in stroke. However, methods to treat glutamate transporter malfunction are limited due to the lack of knowledge of the detailed transport mechanism of these transporters under physiological and pathological conditions. To date no crystal structure of human glutamate transporter is available. All crystal structures of glutamate transporter are from the archaeal glutamate transporter homologue Gltph. Inspection of the available Gltph crystal structures in different states gave rise to two crucial questions that are addressed in our study.

Aim 1: To identify the third Na\(^+\) binding site in glutamate transporter and the role of this Na\(^+\) ion in the transport mechanism. In Gltph crystal structure, only two cation binding sites are visible, representing two Na\(^+\) binding sites. However, three Na\(^+\) ions are cotransported with one substrate in each transport cycle. This raises the question of where the third Na\(^+\) ion binds and what role this Na\(^+\) plays in the transport mechanism. In experiments using voltage clamp fluorometry and simulations based on molecular dynamics combined with grand canonical monte carlo and free energy simulations performed on different iso-forms of Gltph as well on a homology model of EAAT3, we
locate the third sodium binding site in EAAT3. Both experiments and computer simulations suggest that T370 and N451 (T314 and N401 in GltPh) form part of the third sodium-binding site. Interestingly, the sodium bound at T370 forms part of the binding site for the amino acid substrate, perhaps explaining both the strict coupling of sodium transport to uptake of glutamate and the ion selectivity of the affinity for the transported amino acid in EAATs. Results for Aim 1 are presented in Chapter 2.

Aim 2: To study the conformational changes of the human glutamate transporter during the transport cycle in a physiological environment. Crystal structures of the inward and outward facing states of Gltph suggest that there are large rearrangements between the two conformations. In contrast, our previous fluorescence resonance energy transfer (FRET) measurements in human glutamate transporter EAAT3 suggest that only small-scale molecular motions accomplish glutamate uptake. To solve this controversy and further investigate the conformational changes accompanying ion and glutamate transport, we improve the FRET technique by introducing transition metal FRET on EAAT3 transporters expressed in living cell membranes. We find that the extracellular gate of the human glutamate transporter EAAT3 is in the open conformation in the presence of the competitive transporter blocker L-TBOA, corresponding well with L-TBOA bound Gltph crystal structure. In contrast, HP2 is in a relatively closed conformation in Apo, Na\(^+\), K\(^+\), or Na\(^+\) plus glutamate conditions, suggesting that to function as the extracellular gate HP2 does not require as large an opening movement as sterically created by L-TBOA. For the outward/inward conformations of EAAT3, we find that the transporter favors the outward-facing state in the presence of the extracellular competitive transporter blocker L-TBOA, favors more the inward-facing state in K\(^+\),
while the outward-facing and inward-facing states distribution is in an intermediate range in Apo (ion and substrate free), Na⁺, or Na⁺ plus glutamate. Our FRET experiments reveal large conformation changes of the transport domain relative to the trimerization domain during substrate translocation in human glutamate transporter. Interestingly, our FRET result suggests that the Na⁺ bound only transporter in the absence of the substrate glutamate can also isomerize between outward-facing and inward-facing conformations. Results for Aim 2 are presented in Chapter 3.
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Chapter 1

Neurotransmitter transporters: structure meets function

1.1 Summary

At synapses, sodium-coupled transporters remove released neurotransmitters, thereby recycling them and maintaining a low extracellular concentration of the neurotransmitter. The molecular mechanism underlying sodium-coupled neurotransmitter uptake is not completely understood. Several structures of homologues of human neurotransmitter transporters have been solved with X-ray crystallography. These crystal structures have spurred a plethora of computational and experimental work to elucidate the molecular mechanism underlying sodium-coupled transport. Here, we compare the structures of GltPh, a glutamate transporter homologue, and LeuT, a homologue of neurotransmitter transporters for the biogenic amines and inhibitory molecules GABA and glycine. We relate these structures to data obtained from experiments and computational simulations, to draw conclusions about the mechanism of uptake by sodium-coupled neurotransmitter transporters. Here, we propose how sodium and substrate binding is coupled and how binding of sodium and substrate opens and closes the gates in these transporters, thereby leading to an efficient coupled transport.

1.2 Neurotransmitter transporters

Communication between cells in the nervous system is mainly chemical, through presynaptic release of neurotransmitters, diffusion across the synapse, and activation of receptors in the post-synaptic cell (Fig. 1.1A). The released molecules, for example, glutamate, GABA, serotonin, or dopamine, are subsequently removed from the
extracellular space and transported back into the neuron or surrounding glial cells by neurotransmitter transporters. Their removal allows for subsequent release to exert full effect, as well as to localize signaling action to a synapse (Fig. 1.1B). Removal also prevents the prolonged presence of high concentrations of neurotransmitter, which can be detrimental in other ways. For example, high concentrations of extracellular glutamate are neurotoxic - basal extracellular glutamate concentration must be kept low (Danbolt, 2001; Grewer and Rauen, 2005).

Extracellular glutamate is removed from the synapse by transporters called Excitatory Amino Acid Transporters (EAATs), which are expressed in neurons and glia. EAATs belong to solute carrier family 1 (SLC1; Fig. 1.1C). Serotonin, noradrenaline, dopamine, GABA, and glycine are removed by neurotransmitter sodium symporters (NSSs), belonging to solute carrier family 6 (SLC6; Fig. 1.1D). Due to their crucial role of keeping basal concentrations of neurotransmitters low, malfunction or improper regulation of these transporters contributes to neurological and neuropsychiatric disorders (Gether et al., 2006). For example, during ischemia in the brain caused by stroke, EAATs can malfunction and release glutamate, thereby elevating glutamate levels in the extracellular space to neurotoxic levels and causing massive neuronal death (Grewer and Rauen, 2005; Rossi et al., 2000). In addition, many drugs target the transporters, including drugs of abuse, such as cocaine and amphetamine, as well as drugs to treat depression, anxiety, obesity, and epilepsy (Kristensen et al., 2011).
Figure 1.1 Transporter function and family trees. A) A presynaptic action potential (Vpre) causes synaptic release of neurotransmitter that diffuses across the synapse and activates postsynaptic receptors to cause an excitatory postsynaptic potential (Vpost). Subsequently, neurotransmitters diffuse out of the synapse and are taken up by neurotransmitter transporters. B) (left) Neurotransmitter transporters decrease the chance of neurotransmitter spillover by removing neurotransmitters released at one synapse before it has reached a nearby synapse. (right) If neurotransmitter transporters are blocked pharmacologically, then neurotransmitters will have a great chance of reaching nearby synapses and cause a postsynaptic response in nearby synapses (spillover). C) SLC1 family tree containing both aspartate/glutamate and neutral amino acid transporters. D) SLC6 family tree containing amino acid, orphan, monamine, and GABA transporters. For comprehensive descriptions of SLC1 and SLC6 family members, we refer readers to the following excellent reviews (Broer and Gether, 2012; Kanai and Hediger, 2003; Kristensen et al., 2011).

A number of structures of the transporters GltPβ and LeuT, homologous to the mammalian EAATs and NSSs, respectively, have been elucidated in various states by X-ray crystallography (Boudker et al., 2007; Krishnamurthy and Gouaux, 2012; Piscitelli
and Gouaux, 2012; Quick et al., 2009; Reyes et al., 2009; Singh et al., 2008; Singh et al., 2007; Verdon and Boudker, 2012; Wang et al., 2012; Yamashita et al., 2005; Yernool et al., 2004; Zhou et al., 2007; Zhou et al., 2009). In-depth analysis of these structures and other proteins with similar structures have been conducted in several excellent reviews (Abramson and Wright, 2009; Boudker and Verdon, 2010; Forrest et al., 2011; Krishnamurthy et al., 2009). Here, we compare side by side the structures of Glt_Ph and LeuT and relate these structures to data obtained from experiments and computational simulations, to draw conclusions about the mechanism of uptake by neurotransmitter transporters. We focus on how sodium and substrate binding is coupled and how binding of sodium and substrate affects the gates in these transporters, thereby leading to an efficient coupled transport.

1.3 Basic mechanism of secondary active transporters: alternating access

Neurotransmitter transporters are mainly powered by the Na\(^+\) gradient across the plasma membrane. The NSS-type transporters cotransport one to three Na\(^+\) (depending on the specific transporter), most coftransport one Cl\(^-\), and some counter-transport one K\(^+\) or H\(^+\) per substrate molecule transported; reviewed in (Kristensen et al., 2011) (Fig. 1.2A). The EAAT-type transporters co-transport three Na\(^+\) and one H\(^+\), and counter-transport one K\(^+\), for each glutamate molecule (Fig. 1.2A) (Billups et al., 1998; Zerangue and Kavanaugh, 1996). The coupling stoichiometries for the bacterial/archaeal homologues of NSS (LeuT) and EAATs (Glt_Ph) are as follows; LeuT co-transport two Na\(^+\) (Yamashita et al., 2005) and Glt_Ph co-transport three Na\(^+\) (Groeneveld and Slotboom, 2010) per transported substrate molecule (Fig. 1.2A).
How is coupled transport thought to be accomplished by neurotransmitter transporters? The models made for most secondary active transporters involve “alternating access”: a binding site for both substrate and transported ions is alternately accessible either to the external or the internal solution, but never to both solutions at the same time (Mitchell, 1957). Two versions have been proposed for alternating access: the rocker switch (Fig. 2b) (Jardetzky, 1966; Vidaver, 1966) and the two-gated pore (Fig. 1.2C) (Patlak, 1957). In the rocker switch, the transporter is composed of two domains able to undergo a rigid-body rocking motion relative to one another so that external access to the binding site is closed and the internal access to the binding site is simultaneously opened, or vice versa (Fig. 1.2B). In the two-gated pore (Fig. 1.2C), a pore across the membrane is terminated by a gate at each end. Only one gate is open at any time; both can be closed, but both gates cannot be open simultaneously. Binding of substrate and the ions from the exposed side of the membrane closes the gate on that side. The state with both gates closed around the trapped substrate is referred to as the occluded state. From the occluded state, the gate on the opposite side of the membrane can open and allow the substrate and the ions to diffuse out of the pore, thereby completing the coupled transport (Fig. 1.2C). Recent models based on crystal structures of GltPh and LeuT combine aspects of both the rocker switch and the two-gate pore models.
Figure 1.2 Stoichiometry and alternating access of transporters. A) Stoichiometry of uptake of substrate and ions in NSS, LeuT, EAAT, and GltPh transporters in one uptake cycle. LeuT transports small hydrophobic amino acids and GltPh transporters aspartate. B-C) Alternating access mechanism in B) rocker-switch model and C) two-gated pore model. D) Model of co-transport of sodium (Na\(^+\)) and aspartate (asp) in GltPh. E) Model of co-transport of sodium (Na\(^+\)) and glutamate (glu) and counter-transport of potassium (K\(^+\)) in one uptake cycle of EAATs. Only one Na\(^+\) is shown for simplicity in D) and E).

Alternating access can produce either cotransport of ions and substrate (both transported in same direction across membrane, as described above) or countertransport of ions and substrate (ions and substrate transported in opposite directions across membrane), depending on the postulated rules of switching between the outward-facing
to inward-facing conformations. A cotransporter switches from one conformation to the other only when both substrate and coupled ions are bound to the transporter or when neither is bound (Fig. 1.2D). In cotransport, the transporter with either only substrate bound or only the coupled ions bound does not switch between outward and inward conformations, lest it generates a leak flow of substrate or the coupled ions. This leak flow would alter the measured stoichiometry of substrate and coupled ions and degrade the energy coupling for coupled uptake. EAATs have been shown to have an uncoupled Cl⁻ leak current (Wadiche et al., 1995a), but because this current is not coupled to glutamate uptake it does not affect the stoichiometry of coupled uptake. However, some NSSs display Na⁺ leak currents (Lester et al., 1996) and these would be predicted to degrade the energy for coupled uptake in NSS uptake. A transporter (such as in Fig. 1.2C) functions as a countertransporter if it can switch between the outward- and inward-facing conformations only when either substrate or ions are bound to the transporter, but not when both substrate and ions are bound or when neither is bound to the transporter (Fig. 2E). More complicated models for co- and countertransport are possible, but, for example, the EAATs seem to use these simple rules to co-transport Na⁺/H⁺/glutamate and countertransport K⁺ using two gates that control access to a binding pocket (Fig. 2E).

The transport models can accomplish substrate uptake into the cell by clockwise stepping through the states of the cycle, or substrate release by counter-clockwise stepping through the states (Fig. 1.2D and 1.2E). In which direction does the transport normally occur? The requirement for net secondary active transport is that the free energy drop in transporting the coupled ions down their electro-chemical gradient exceeds the free energy required for transporting the substrate against its electro-chemical gradient.
An EAAT transporter operating under physiological ionic conditions and voltage is thought to be able to establish a transmembrane glutamate concentration ratio of $10^6$ (10 mM inside/10 nM outside the cell) (Zerangue and Kavanaugh, 1996). The direction of transport or maximal gradient achieved by the transporter is only determined by the stoichiometry of the substrate and transported ions and the thermodynamic gradient for transport, and not the molecular details of transport or the affinity for the different molecules in the different states (but these factors could determine the kinetics of transport).

So what is the structural basis for how neurotransmitter transporters implement alternating access and accomplish a coupled co- or countertransport necessary for an efficient uptake of neurotransmitters against steep concentration gradients of neurotransmitters? In the next section, we review which states in the transport cycle have been identified for Glt$_{Ph}$ and LeuT.

### 1.4 Crystallographic structures of archaeal/bacterial homologues

Atomic resolution 3D structural information on sodium-coupled neurotransmitter transporters started to arrive in 2004 and 2005 with reports of the structures of Glt$_{Ph}$, an archaeal EAAT homologue from *Pyrococcus horikoshii* (Yernool et al., 2004), and LeuT, a bacterial NSS homologue from *Aquifex aeolicus* (Yamashita et al., 2005). Structures of these transporters have now been determined in various conformations, beginning to reveal the structural basis of substrate and ion binding, mechanisms of inhibition, and mechanisms of transport.
Figure 1.3 Structural makeup of Glt\textsubscript{Ph} and LeuT. A) Glt\textsubscript{Ph} assembles as a bowl-shaped trimer. Left, extracellular view. Right, view parallel to the membrane. Individual monomers are colored wheat, blue, green. B) LeuT monomer, viewed parallel to the membrane. C) Primary structure of a Glt\textsubscript{Ph} monomer. First inverted repeat (AA\textsuperscript{1}: blue, yellow) and second inverted repeat (BB\textsuperscript{1}: magenta, green) displayed as triangles. D) Structural relationship of internal repeat structures in Glt\textsubscript{Ph}. Scaffold domain, left. Core domain, middle. Protomer fold, right. TMs colored as in C). E) Primary structure of LeuT. Inverted repeat defined by gray shaded area. TMs 1-2 (A: magenta) and 6-7 (A\textsuperscript{1}: green), as well as TMs 3-5 (B: blue) and 8-10 (B\textsuperscript{1}: yellow), are symmetrically related. F) Structural relationship of internal repeat structures in LeuT. Scaffold domain, left. Core domain, middle. Protomer fold, right. TMs colored as in E). TMs 11 and 12 shown only in the protomer fold for clarity.
The first published structures revealed substantial differences in the three-dimensional fold of Glt\(_{Ph}\) (Fig. 1.3A) and LeuT (Fig. 1.3B), yet presented a common theme of two-fold internal structural symmetry and discontinuous membrane helices (Yamashita et al., 2005; Yernool et al., 2004). Glt\(_{Ph}\) assembles as a bowl-shaped trimer with a large solvent-filled basin open to the extracellular solution (Fig. 1.3A) (Yernool et al., 2004). Each protomer in Glt\(_{Ph}\) is made up of two sets of inverted repeats (AA\(^{-1}\)BB\(^{-1}\): Fig. 1.3C). The first inverted repeat forms a scaffold domain consisting of six transmembrane segments (TM1-6) folded into a cylinder that houses the second repeat; a core domain (Fig. 1.3D) consisting of two reentrant helical hairpin loops (HP1 and HP2) and two transmembrane helices (TM7 and TM8) (Fig. 1.3C).

In contrast, LeuT is a monomer made up of 12 transmembrane helices and with a fold resembling a shallow “shot glass” (Fig. 1.3E and 1.3F) (Yamashita et al., 2005). In LeuT, the first ten transmembrane helices constitute an internal structural repeat relating the first five helices to the second five by a pseudo-two-fold axis parallel to the membrane plane (ABA\(^{-1}\)B\(^{-1}\): Fig. 1.3E). TMs 1, 2, 6, and 7 form a centrally located core domain, while TMs 3-5 and 8-10 form a surrounding scaffold domain (Fig. 1.3F). For both Glt\(_{Ph}\) and LeuT, the inverted repeat structural elements were not a priori predicted from sequence analysis, but only apparent in the crystal structures. The inverted repeats have proven integral to understanding the basic transport mechanisms of Glt\(_{Ph}\) and LeuT and are a common theme for many transporters with different architectures (Abramson and Wright, 2009; Boudker and Verdon, 2010; Forrest et al., 2011; Forrest and Rudnick, 2009; Krishnamurthy et al., 2009). Furthermore, the LeuT fold itself has been found in a number of seemingly unrelated transporters, establishing the two-fold-related “5+5”
transmembrane repeat as an integral aspect of numerous transporters (Abramson and Wright, 2009; Boudker and Verdon, 2010; Forrest et al., 2011; Forrest and Rudnick, 2009; Krishnamurthy et al., 2009), while the Glt\textsubscript{Ph}-like folds are quite rare by comparison (Johnson et al., 2012).

1.4.1 Substrate and ion-bound “outward-occluded” states

In the first published structures of Glt\textsubscript{Ph} and LeuT, the substrates were occluded from solution on both sides of the membrane (Fig. 1.4) (Yamashita et al., 2005; Yernool et al., 2004). In Glt\textsubscript{Ph}, the substrate was occluded by HP2 on the extracellular side (Fig. 4A and 5B), suggesting HP2 forms the extracellular gate (Yernool et al., 2004). In LeuT, Tyr 108 and Phe 253 sequestered the substrate and ion binding sites from the extracellular solution. These residues, together with residues Arg 30 and Asp 404 and the extracellular loop 4 (EL4), were proposed to form the extracellular gate in LeuT (Fig. 1.4B & 1.5B) (Yamashita et al., 2005). Additionally, in both structures the proposed extracellular gates were observed to be “thin” sections of protein, while access to the substrate and ion-binding sites from the intracellular side of the membrane was obstructed by 15-20 Å of “thick” sections of protein (Fig. 1.4) (Krishnamurthy et al., 2009; Yamashita et al., 2005; Yernool et al., 2004). These crystal structures were thus proposed to represent outward-facing occluded states of the transporters with both gates closed.
1.4.2 Mechanisms of inhibition and gating

Direct evidence supporting the proposed nature of the extracellular gates in Glt\textsubscript{Ph} and LeuT has been provided by crystal structures solved in complex with various inhibitors. The crystal structure of Glt\textsubscript{Ph} in complex with the non-transportable, competitive inhibitor D,L-threo-\(\beta\)-benzyloxyaspartate (TBOA) revealed a structure in which the tip of HP2 was displaced by about 10 Å (Fig. 1.5A) from its position in an aspartate-bound structure (Fig. 1.5B) (Boudker et al., 2007). Displacement of HP2 was
found to be due to a steric hindrance of HP2 induced by the bulky benzyl group of TBOA (Fig. 1.5A), with the aspartate moiety of TBOA residing in the substrate-binding pocket (Boudker et al., 2007). The structural basis of TBOA-inhibition therefore was revealed to be a result of competitive inhibition, locking Glt_{Ph} in an outward-facing state with the extracellular gate (HP2) propped open.

Like TBOA inhibition of Glt_{Ph}, the competitive inhibitor tryptophan inhibits LeuT by displacing the substrate and trapping the transporter in an outward-facing conformation with the extracellular gate locked open (Fig. 1.5A) (Singh et al., 2008). This conformation is largely the result of outward rotation of TMs 1b, 6a, and EL4, and an increase in the distance between the extracellular gate residues Y108 and F253 (cf. Fig. 5A and 5B) (Singh et al., 2008). Together these movements result in a widening of the extracellular vestibule and increased solvent accessibility to the substrate-binding site.

In contrast, LeuT crystal structures in complex with tricyclic antidepressants (TCAs) (Singh et al., 2007; Zhou et al., 2007), selective serotonin re-uptake inhibitors (SSRIs) (Zhou et al., 2009), and the detergent octylglucoside (Quick et al., 2009), have all revealed a non-competitive mechanism of transport inhibition. This non-competitive mechanism has been shown to be the result of the bound inhibitor stabilizing the substrate-bound transporter in an outward-facing occluded conformation with the extracellular gate closed (Singh et al., 2007) (Zhou et al., 2007). Taken together, the mechanism for competitive inhibition in Glt_{Ph} and LeuT appears to be the result of preventing closure of the extracellular gate, while non-competitive inhibitors (evidenced for LeuT alone) function by trapping substrate-bound transporters in an outward-facing occluded state with the extracellular gate closed.
Figure 1.5 Crystal structures of multiple states in GltPh and LeuT. A) D,L-TBOA locks GltPh in an outward-facing state by preventing closure of HP2. Tryptophan locks LeuT in an outward-facing state by increasing the distance between aromatic and charged extracellular gating residues. TMs 1a, 6b, and EL4 are outwardly rotated in the presence of tryptophan, widening the extracellular cavity. B) In the outward occluded state of GltPh, substrate is trapped between HP1 and HP2. In the outward occluded state of LeuT, substrate is blocked from the extracellular solution by the extracellular gate comprised of aromatic and charged amino acids, and EL4. C) In the inward-facing occluded state of GltPh, the core domain is moved towards the cytosol, with substrate remaining trapped between HP1 and HP2. D) The inward-open state of LeuT is the result of an inward tilt of TMs 1b and 6a, inwardly directed movement of EL4, and outward movement of TM1a. No crystal structures have been solved for the inward-occluded state of LeuT or the inward-open state of GltPh (indicated by ?).
1.4.3 Transport principles revealed by inward-occluded Glt\textsubscript{Ph} and inward-open LeuT states

Insight into the conformational changes from the outward- to inward-facing states came from crystal structures of a crosslinked double cysteine Glt\textsubscript{Ph} mutant in the inward-facing occluded state (Reyes et al., 2009) and a mutated LeuT in the inward-facing open state (Krishnamurthy and Gouaux, 2012).

The strategy for Glt\textsubscript{Ph} was based on previous experimental evidence for the formation of a spontaneous intramolecular disulphide bond between two residues in EAAT1 (Ryan et al., 2004). The homologous positions in Glt\textsubscript{Ph} (K55 and A364) were observed to be greater than 25 Å apart in both the outward-open and outward-occluded (Boudker et al., 2007; Yernool et al., 2004) Glt\textsubscript{Ph} structures, suggesting that a large movement was required for these residues to come into close proximity. Crystals obtained following crosslinking K55C and A364C with Hg\textsuperscript{2+} yielded a structure in which the substrate binding site was observed to move approximately 20 Å from its position in the outward-facing structures to a position near the cytoplasm (Fig. 1.5C and 1.6A) (Reyes et al., 2009). In the crosslinked structure, bound L-asp and Na\textsuperscript{+} were observed to be close to the intracellular solution, occluded from the intracellular solution by a “thin” section of protein while occluded from the extracellular solution by a “thick” section of protein (Fig. 1.6A) (Reyes et al., 2009). This structure was thus interpreted as representing an inward-facing occluded state, with both the extracellular and intracellular gates closed. Superimposition of the outward-open, outward-occluded, and inward-occluded states revealed that TM1, TM2, TM4, and TM5 are invariant in position (Reyes et al., 2009). In contrast, the other parts of the protein, housing the substrate and ion
binding sites (TM3, TM6, HP1, TM7, HP2, and TM8), undergo substantial conformational change. Glt$_{\text{Ph}}$ was thus proposed to comprise two structural domains, the ‘trimerization’ domain (TM1, TM2, TM4, TM5) and the ‘transport’ domain (TM3, TM6, HP1, TM7, HP2, and TM8) (Reyes et al., 2009). The trimerization domain consists mainly of the first internal repeat, whereas the transport domain comprises mainly the second internal repeat. This structure suggests that the transition between the outward facing state to the inward facing state involves movement of the transport domain within the frame of a rigid trimerization domain (Fig. 1.6A) (Reyes et al., 2009). No crystal structure of the inward-facing open state of Glt$_{\text{Ph}}$ has been published, leaving it unclear how ions and substrate obtain access to the cytoplasm (but see below for modeling studies).

At present, no crystal structure defines a LeuT inward-facing occluded state. However, recently a LeuT structure in the inward-facing open state was published (Fig. 1.5D) (Krishnamurthy and Gouaux, 2012). Comparison of LeuT structures in the outward-open and outward-occluded states with the inward-open state indicates that the conformational changes are not strictly those of a “rocker switch” (Fig. 1.6B) (Krishnamurthy and Gouaux, 2012). The core domain does not move as a rigid body, as in a rocking bundle mechanism. Instead, only a portion of the core moves as a unit, so that symmetry of the inward- and outward-facing conformations is not strictly preserved (Fig. 1.6B). The transport mechanism in LeuT thus appears to exploit local hinge-like movements that lead to the coordinated opening and closing of both “thin” and “thick” extracellular and intracellular gates (Fig. 1.6B). In contrast, crystal structures of other transporters with the LeuT fold in outward- and inward-facing states are suggestive of a
stricter “rocker switch” mechanism. The adherence to a strict “rocker switch” mechanism or a more local hinge-like mechanism likely depends on the individual transporter in question (Abramson and Wright, 2009; Boudker and Verdon, 2010; Forrest et al., 2011; Forrest and Rudnick, 2009; Jeschke, 2012; Krishnamurthy et al., 2009)

Figure 1.6 Models of the outward-inward transition in GltPh and LeuT. A) The transition from the outward-occluded to inward-occluded state in GltPh involves coordinated movement of the transport domain, which leads to a swap of the “thin” and “thick” gates. Shown for clarity is only the core of GltPh as depicted in Fig. 1.3. B) The transition from the outward-open to inward-open state of LeuT involves a coordinated tilt of TMs 1b and 6a, inwardly directed movement of EL4, and uncoupled outward movement of TM1a. Color scheme is as in Fig. 1.3. Blue areas in (B) indicate water pathways.
Remarkably, models similar to the crystals structures for the inward-facing states of Glt\textsubscript{Ph} and LeuT were generated independently of the crystal structures by utilizing the inverted-topology repeats in Glt\textsubscript{Ph} and LeuT and assuming that the inward-facing states could be modeled by threading the sequence of the first part of each repeat on the structure of second part of each repeat and vice versa (Crisman et al., 2009; Forrest et al., 2008).

The crystal structures of transporters in different states indicate what type of conformational changes the transporters undergo. However, one also needs to understand the principles by which the binding of ions and substrate are coupled to these conformational changes. In this review, we focus on how binding of substrate and Na\textsuperscript{+} are coupled to the conformational changes in the transport cycle. On the role of other ions in neurotransmitter transporters, see a number of excellent reviews (Danbolt, 2001; Grewer and Rauen, 2005; Kristensen et al., 2011).

1.5 Substrate and ion binding

1.5.1 Substrate

In a high-resolution structure of Glt\textsubscript{Ph} crystallized in the presence of L-aspartate (Boudker et al., 2007), aspartate is located deep in the transport domain, between the tips of HP1 and HP2, the non \(\alpha\)-helix region of TM7, and polar residues of TM8 (Fig. 1.7A). Several residues that contribute to the substrate binding site in this Glt\textsubscript{Ph} structure have also been implicated in substrate binding by mammalian EAATs. For example, R397 interacts with the \(\beta\) carboxylate of aspartate in the crystal structures of Glt\textsubscript{Ph} (Fig. 1.7A) (Boudker et al., 2007). The homologous R447 in EAATs has been suggested to interact
with the γ carboxylate of the acidic substrate (Bendahan et al., 2000). Interestingly, the residue homologous to R447 is a neutral residue in the related ASCT neutral amino acid transporters (Bendahan et al., 2000; Boudker et al., 2007).

**Figure 1.7 Substrate and sodium binding sites in GltPh and LeuT.** A) Aspartate and two thallium ions in the crystal structure of GltPh. B) Potential Na⁺ sites in simulations of GltPh. C) Leucine and two Na⁺ in the crystal structure of LeuT. D) Two Na⁺ in LeuT. Leucine removed for clarity. Side chains and backbones interacting with substrate and cations are shown in A)-D) as stick.

All LeuT structures with bound substrate show a substrate molecule accommodated in a common site (termed S1) together with two Na⁺ ions (Fig. 1.7C) (Yamashita et al., 2005). The substrate and the two Na⁺ are located in a central cavity formed by a four-helix bundle comprised of TM1, TM3, TM6, and TM8 (Yamashita et
In the substrate binding site the polar α-amino and α-carboxylate groups of the amino acid substrate interacts with a phenolic hydroxyl moiety from Tyr108 in TM3, one Na\(^+\), and the unwound regions of TM1 and TM6. The substrate is stabilized by hydrogen bonds with the backbone of TM1 and TM6 and by interacting with the ends of these α helices (Fig. 1.7C). The hydrophobic side chain of the substrate is accommodated by hydrophobic side chains from TM3, TM6, and TM8. The residues forming the S1 substrate binding site are conserved between LeuT and the NSS family of transporters and several S1 residues are important for substrate selectivity and affinity in NSS transporters (Kristensen et al., 2011), suggesting that the S1 site is conserved in NSS transporters.

Comparison of the substrate binding sites in Glt\(_{Ph}\) and LeuT shows that the substrate binding site in Glt\(_{Ph}\) is entirely within the core of the transport domain, whereas in LeuT the substrate binding site is on the interface between the transport and scaffold domains. But, Glt\(_{Ph}\) and LeuT share commonalities in the way the substrates are bound, including the heavy involvement of the non-helical regions of the trans-membrane segments and involvement of backbone polar groups in substrate coordination.

### 1.5.2 Na\(^+\)

Na\(^+\) was replaced with Tl\(^+\) in a Glt\(_{Ph}\) crystal structure for its higher anomalous scattering signal (Boudker et al., 2007). Two Tl\(^+\) ions observed bound to Glt\(_{Ph}\) were labeled Na1 and Na2 (Boudker et al., 2007). Na1 is buried deeply in the transporter structure and is mainly coordinated by residue D405 in TM8 (Fig. 1.7A). Na2 is coordinated by backbone oxygen atoms of the tip of HP2 and the unwound section in the center of TM7, close to extracellular solution (Fig. 1.7A). Experimental studies lend
support that the two Tl⁺ in the crystal structure represent Na⁺ binding sites (Boudker et al., 2007; Tao et al., 2008; Teichman et al., 2009).

However, three Na⁺ are cotransported with one substrate in each Gltₚhs transport cycle (Groeneveld and Slotboom, 2010), similar to EAATs (Zerangue and Kavanaugh, 1996). This raises the question of where the third Na⁺ ion binds and what role this Na⁺ plays in the transport mechanism. Computations and experiments have not yet produced a consensus regarding the location of the third Na⁺ site (Bastug et al., 2012; Bendahan et al., 2000; Huang et al., 2009; Larsson et al., 2010; Rosental et al., 2006; Ryan et al., 2009; Shrivastava et al., 2008; Tao et al., 2010; Teichman et al., 2012). For example D312 in TM7 and T92 in TM3 have been suggested to form a Na3 site that binds Na⁺ prior to substrate binding (Fig. 1.7B) (Bastug et al., 2012; Huang and Tajkhorshid, 2010; Tao et al., 2010). Another study proposed this site to be a transiently occupied site (called Na3’), because a Na⁺ bound at this location was electrostatically destabilized by a Na⁺ bound at Na1 site (Larsson et al., 2010). This study proposed another Na3 site, involving the side chains of T314 and N401 and, interestingly, the charged β-carboxylate group of the bound substrate (Fig. 1.7B) (Larsson et al., 2010). Direct substrate-ion contact had been previously suggested by electrostatic mapping (Holley and Kavanaugh, 2009) and is similar to what has been found in LeuT (Yamashita et al., 2005). Direct contact of Na3 with the substrate could explain the observation that the apparent affinity for different acidic amino acids depends on the nature of the cotransported cation (Menaker et al., 2006) and the findings that mutants of residues homologous to N401 and T314 both alter cation and substrate selectivity (Larsson et al., 2010; Teichman et al., 2012).
With evidence for more Na\(^+\) sites than the number of transported Na\(^+\), we propose that the three transported Na\(^+\) go through one or more intermediate binding sites before finalizing their binding positions. Consistent with this idea, another site, formed by a conserved aspartate residue together with a tyrosine and located at the external end of the binding pocket, is proposed to be a transient Na\(^+\) site, through which one or more of the Na\(^+\) have to pass on their way to other binding sites (Rosental et al., 2011). To further determine the location and function of the different proposed Na\(^+\) binding sites additional functional and/or computational approaches are required, including techniques allowing for manipulation of the backbone carbonyls which contribute extensively to Na\(^+\) binding in Glt\(_{PB}\) and likely in EAATs.

Structures of LeuT obtained in the presence of substrate and Na\(^+\) reveal two distinct Na\(^+\) sites, termed Na1 and Na2, in the S1 binding pocket (Yamashita et al., 2005). The Na1 and Na2 sites coordinate Na\(^+\) by six and five oxygen ligands, respectively. (Fig. 1.7D). The Na1 site is highly conserved among the NSS family. In the Na2 site, residues G20, V23, A351 and S355 in TM1 and TM8 are also well conserved in eukaryotic SLC6 transporters. The Na\(^+\) at Na1 interacts directly with the substrate carboxyl group and, according to computer simulations (Noskov et al., 2008), the strong electrostatic field generated by the carboxylate of leucine appears to control the selectivity for Na\(^+\) at the Na1 site. Na1 is suggested to bind Na\(^+\) before substrate binding (Yamashita et al., 2005). Consistent with this binding order, in a recent Na\(^+\)-bound, substrate-free structure of LeuT in which the “thin” extracellular gate is open, Na\(^+\)-binding at Na1 is observed to impart a stabilizing effect on TM5 1b and 6a, proposed to be an important aspect for subsequent substrate-binding (Krishnamurthy and Gouaux, 2012). Selectivity for Na2
seems to be driven by the local structure constraints of the cavity created by the five neutral coordinating oxygen ligands (Noskov et al., 2008). Although Na\(^+\) binding sites similar to Na1 and Na2 exist in the mammalian NSS transporters, the precise role of Na\(^+\) in mammalian NSSs. More functional data addressing the role of each site in the individual NSS members is needed. Interestingly, in other sodium-coupled transporters that belong to different families (other than NSS) and have low overall sequence similarity, but share the LeuT-type fold, biochemical and structural studies have identified Na2 as being highly conserved, whereas Na1 is less conserved (Khafizov et al., 2012). This suggests that sodium binding to Na2 is integral to the sodium-coupling mechanism in transporters with the LeuT-type fold (see below for more details on possible role of Na2).

The amino acid sequence and three-dimensional structures of Glt\(_{Ph}\) and LeuT are unrelated, yet they share similarities in the local protein organization and the coordination of the Na\(^+\) sites (Boudker et al., 2007). This suggests that Na\(^+\)-dependent transporters might possess a common Na\(^+\)-binding motif, for example the unwound transmembrane segments of Glt\(_{Ph}\) (TM7) and LeuT (TM1) (Boudker et al., 2007). In addition, both Na1 and Na2 are formed, in part, by coordinating carbonyl oxygen atoms occupying nearly equivalent positions in Glt\(_{Ph}\) and LeuT.

**1.6 Alternating access model meets structure**

Starting from crystal structures of Glt\(_{Ph}\) and LeuT obtained in various conformations, both computational studies and structure-based experiments are beginning to uncover the dynamics of the transport by EAAT and NSS. We here focus on how Na\(^+\) and substrate
binding are coupled to the gates of the transporter and the transitions in the transport cycle.

1.6.1 Dynamics of the extracellular gate

If a co-transporter with either only substrate or ions bound is allowed to switch from outward-to-inward facing (Fig. 1.2D and 1.2E), it would allow a leakage, with consequences for the stoichiometry and energy efficiency of transport. In both EAATs and NSSs, the binding of at least one Na\(^+\) precedes the binding of substrate (Grewer and Rauen, 2005; Krishnamurthy and Gouaux, 2012; Larsson et al., 2004). What are the structural implications of Na\(^+\)-binding prior to substrate binding? As mentioned above, in both LeuT and Glt\(_{Ph}\) one of the Na\(^+\) seems to form part of the substrate binding site. In addition, Na\(^+\) binding also seems to have effects on the gates, in ways that prevent leakage.

Molecular dynamics simulations of Glt\(_{Ph}\) (Huang and Tajkhorshid, 2008; Shrivastava et al., 2008) showed fluctuations of the extracellular gate (HP2) between the closed and open conformations in the apo-state and in the presence of Na\(^+\) alone without substrate (Huang and Tajkhorshid, 2008). However, electron paramagnetic resonance experiments on spin-labeled Glt\(_{Ph}\) suggested that in the presence of Na\(^+\) HP2 is biased toward a more open conformation, whereas in the apo state HP2 occupies a more closed conformation (Focke et al., 2011), consistent with crosslinking studies on Glt\(_{Ph}\) in the apo-state (Reyes et al., 2009). A recent simulation study suggested that the binding of Na\(^+\) stabilizes a state of HP2 intermediate between open and closed (Grazioso et al., 2012). A possible interpretation of these experiments and simulations is that HP2 fluctuates between closed and open. In the apo-state, HP2 is biased towards the closed
state and, in the Na\(^+\)-bound state, HP2 is biased towards an open state (Fig. 1.8A). The stabilization of HP2 in the open state (Fig. 1.8A) would prevent isomerization to the inward-facing state in the presence of Na\(^+\) alone (in the absence of substrate). Interestingly, similar findings have been reported for LeuT. For LeuT in the apo-state, simulations show fluctuations in the extracellular gate (Celik et al., 2008; Claxton et al., 2010), with a bias for the inward-facing state (Shi et al., 2008). The binding of Na\(^+\) in the absence of substrate has been shown to promote an outward-open conformation of the extracellular gate of LeuT (Fig. 1.8B) (Celik et al., 2008; Claxton et al., 2010; Shi et al., 2008). A recent crystal structure of LeuT in the presence of Na\(^+\) alone, in which the extracellular gate is open, further supports the idea that Na\(^+\) binding stabilizes the open gate (Krishnamurthy and Gouaux, 2012).

This model is a structural correlate to the model of cotransport in which isomerization of the transporter from an outward- to an inward-facing state is prevented when only Na\(^+\) is bound (Fig. 1.8). The subsequent binding of substrate (and additional ions) is predicted to bias the transporter toward a closed conformation (Fig. 1.8). Indeed, in Glt\(_{Ph}\), substrate (aspartate) binding is observed computationally and experimentally to induce a closure of the extracellular gate (Focke et al., 2011; Grazioso et al., 2012; Huang and Tajkhorshid, 2008), with Na\(^+\) binding to the last Na2 site in the presence of substrate is predicted to stabilize the closed state (Fig. 1.8A) (Grazioso et al., 2012; Huang and Tajkhorshid, 2008; Shrivastava et al., 2008). Similarly, in LeuT, substrate binding in the presence of Na\(^+\) shifts the conformation from an outward-facing open state to an outward-facing occluded state (Fig. 1.8B), reducing extracellular gate fluctuations and
preventing access to the binding sites (Celik et al., 2008; Claxton et al., 2010; Shi et al., 2008).

**Figure 1.8 Models of the transport mechanism in GltPh and LeuT.** In the outward-facing apo state (1) external gate movements (HP2 in GltPh and TMs1b, 6a and EL4 in LeuT) allow for sodium binding, which stabilizes an outward-facing open state (2). This sets up a binding site for substrate and additional ion(s), leading to formation of an outward-occluded state (3). Transition to an inward-facing occluded state (4) involves piston-like motion of the transport domain (magenta and green) relative to the trimerization domain (grey) in GltPh and inwardly-directed movement of EL4 and a coordinated tilt of TMs 1b,6a in LeuT. Release of the first sodium (Na2) leads to opening of the intracellular gate (HP1 in GltPh and TM1a in LeuT) (5) and subsequent release of substrate and additional ion(s). In the inward-facing apo-state (6), the intracellular gate closes to allow for transition to the outward-facing apo-state.
1.6.2 Outward-to-inward facing transition

In both GltPh and LeuT, the transition between the outward- and inward-facing conformation is largely due to the movements of the core domain relative to the scaffold domain (Figs. 1.6 & 1.8).

Computational studies in GltPh suggest that the transition from the outward facing to the inward facing state involves unequal movements of the trimerization and transport domain in opposite directions, together with a tilt in the transport domain with respect to the membrane (Stolzenberg et al., 2012). These motions result in significant changes in contacts between the trimerization and transport domains and are in line with a recent crystal structure of GltPh captured in an intermediate state (Verdon and Boudker, 2012). This study also observed a conformational change involving the TM3-4 loop during the transport process, consistent with a recent experimental study that suggested that the TM3-4 loop is essential for transport in GltPh (Compton et al., 2010). Another simulation study (Lezon and Bahar, 2012) suggests that the lateral pressure of the membrane on the transporter help guide the protein in the inward-to-outward isomerization in GltPh. Consistent with experimental evidence in EAATs and GltPh crystal structures, both simulation studies observed structural asymmetry of the different subunits, providing further evidence of the independent nature of individual subunits (Grewer et al., 2005; Groeneveld and Slotboom, 2007; Koch et al., 2007; Koch and Larsson, 2005; Leary et al., 2007). In summary, the modeling studies on GltPh are consistent with the large piston-like movement of the transport domain with respect to the trimerization domain in the transition between outward-facing to inward-facing states (Figs. 1.6A & 1.8A) (Reyes et al., 2009).
For LeuT, the transition from outward-to-inward facing is more complicated and controversial. A model for the inward-facing state of LeuT based on structural repeats initially suggested the possibility of a “rocking bundle” mechanism of alternating access (Forrest et al., 2008). However, recent crystal structures suggest that LeuT may not strictly adhere to a rocking bundle mechanism, but that the transition from outward-to-inward involves a combination of rocking bundle and two-gated pore models (Fig. 6B & 8B) (Krishnamurthy and Gouaux, 2012). In addition, computational and experimental studies have proposed that the binding of a second substrate molecule at a secondary site (S2) is required for the outward-to-inward transition and subsequent substrate and ion release (Shan et al., 2011; Shi et al., 2008; Zhao et al., 2010; Zhao et al., 2011). At the present time it is unclear whether binding at the S2 site is necessary or not (Piscitelli and Gouaux, 2012; Quick et al., 2012; Wang et al., 2012). Nonetheless, recent reports point to a complex transport mechanism: a combination of local conformational change and rigid body movements in LeuT (Krishnamurthy and Gouaux, 2012; Shaikh and Tajkhorshid, 2010; Zhao and Noskov, 2011; Zhao et al., 2011).

1.6.3 Dynamics of the intracellular gate

How are ions and substrate released to the cytoplasm in GltPh and LeuT? The inward facing crystal structure of GltPh is in an occluded state (Reyes et al., 2009), with no access of the substrate to the cytosol. A computational model of the inward-facing open state, in which HP1 is assumed to be the intracellular gate, was generated based on the TBOA-bound structure of GltPh and the assumption that HP1 in the inward-facing open state adopts the same conformation as HP2 in the TBOA-bound outward-facing open state (Crisman et al., 2009). Two recent simulation studies point to the dissociation
of Na2 as the trigger for intracellular release (DeChancie et al., 2011; Grazioso et al., 2012). Following dissociation of Na2, HP1 is observed to allow water molecules to enter the region and initiate release of the substrate and ions via an increase in the flexibility of HP1 (Fig. 8A). Following the release of Na1, HP1 undergoes large fluctuations similar to what is observed for HP2 in the outward-facing apo-state. These fluctuations in HP1 are hypothesized to allow HP1 to close and thereby enable the binding pocket to translocate back across the membrane (Fig. 1.8A), consistent with a proposal for GltPh based on EPR measurements (Focke et al., 2011).

The structure of LeuT in the inward-facing open state suggests a potential mechanism of how ion release is coupled to transporter opening to the cytoplasm: the release of Na2 and a conformational change involving hinge movement in TM1a create conditions favorable for subsequent substrate and ion release (Figs. 1.6B & 1.8B). Computational models of the inward-facing state(s) supports that hydration of Na2 play a prominent role in the substrate/ion release in LeuT, similar to the proposed role of Na2 in substrate release in GltPh (Shaikh and Tajkhorshid, 2010; Zhao and Noskov, 2011).

1.7 General mechanisms

Even if crystal structures show structural differences between GltPh and LeuT, we think that general mechanistic principles shared by sodium-coupled neurotransmitter transporters can be drawn from GltPh and LeuT (Fig. 1.8). We propose the following general mechanism for sodium-coupled neurotransmitter uptake. In the outward-facing apo state, the extracellular gate fluctuates, allowing access for Na\textsuperscript{+} to its binding sites. The binding of extracellular Na\textsuperscript{+} to the transporter stabilizes the outward-facing open
state, preventing Na\(^+\) leakage and creating a high-affinity substrate-binding site. At least one of the bound Na\(^+\) seems to become part of the substrate binding site in both Glt\(_{ph}\) and LeuT. Following substrate (and potentially additional ion) binding, the extracellular gate closes and a transition to the inward-facing occluded state occurs. In Glt\(_{ph}\) this process involves a piston-like movement of the transport domain with respect to the trimerization domain, whereas in LeuT the core domain undergoes a combination of rocking bundle and local hinge movements with respect to the scaffold domain. In the inward-facing state, the initial event triggering substrate release appears to be the release of Na\(_2\). Following release of Na\(_2\), the closed state of the intracellular gate is destabilized, leading to release of further ions and substrate. In both Glt\(_{ph}\) and LeuT, we propose that in the inward-facing apo-state the intracellular gate continuously opens and closes, resulting in the ability of the intracellular gate to close so that the transporter can transit from the inward-to-outward facing state and, thereby, complete the transport cycle (Fig. 1.8).

1.8 Conclusions (future considerations)
Recent crystal structures of bacterial transporter homologues and recent functional and computational studies of neurotransmitter transporters and their archaeal/bacterial homologues have generated hypotheses for how the EAATs and the NSS transporters accomplish Na\(^+\)-coupled neurotransmitter uptake. However, not all states in the transporter cycles have yet been identified in the archaeal/bacterial transporters, and still no crystal structure is available for any eukaryotic neurotransmitter transporter. In addition, many of the states proposed from existing crystal structures still need to be verified by other methods, such as EPR and FRET. Controversies have erupted in both
the fields of NSS and EAATs. For example, which site is the real third Na$^+$ site in EAATs? And, are there one or two substrate sites in NSS transporters? More crystal structures might help in solving these controversies. But, also longer molecular dynamic simulations (and other computer simulation techniques) of transporters, as well as structural measurements from for example DEER EPR and new FRET techniques, will help in generating models of the complete transport cycle in these transporters. Finally, knowledge of the different states in the transporter cycles at a molecular level will help in developing new drugs that target neurotransmitter transporters to cure or alleviate disorders and diseases.
Chapter 2

Evidence for a third sodium binding site in glutamate transporters suggests an ion/substrate coupling model

2.1 Summary

Excitatory Amino Acid Transporters (EAATs) remove glutamate from synapses. They maintain an efficient synaptic transmission and prevent glutamate from reaching neurotoxic levels. Glutamate transporters couple the uptake of one glutamate to the cotransport of three sodium ions and one proton and the counter-transport of one potassium ion. The molecular mechanism for this coupled uptake of glutamate and its co- and counter-transported ions is not known. In a crystal structure of the bacterial glutamate transporter homologue, GltPh, only two cations are bound to the transporter, and there is no indication of the location of the third sodium site. In experiments using voltage clamp fluorometry and simulations based on molecular dynamics combined with grand canonical monte carlo and free energy simulations performed on different iso-forms of GltPh as well on a homology model of EAAT3, we sought to locate the third sodium binding site in EAAT3. Both experiments and computer simulations suggest that T370 and N451 (T314 and N401 in GltPh) form part of the third sodium-binding site. Interestingly, the sodium bound at T370 forms part of the binding site for the amino acid substrate, perhaps explaining both the strict coupling of sodium transport to uptake of glutamate and the ion selectivity of the affinity for the transported amino acid in EAATs.

2.2 Introductory remarks

Glutamate, the main excitatory neurotransmitter in the central nervous system, is removed from the extracellular synaptic space by the glutamate excitatory amino acid
transporters EAAT1-5 (Danbolt, 2001; Tzingounis and Wadiche, 2007). These transporters thereby maintain an efficient synaptic communication between neurons and prevent extracellular glutamate from reaching neurotoxic levels (Danbolt, 2001; Tzingounis and Wadiche, 2007). EAATs are trimeric proteins in which each subunit functions as an independent transporter (Grewer et al., 2005; Koch and Larsson, 2005). Each subunit has 8 transmembrane domains and two membrane inserted hair-pin (HP) loops (Grunewald et al., 1998; Yernool et al., 2003; Yernool et al., 2004). EAATs use the Na$^+$ and K$^+$ gradients in driving the uptake of glutamate against a concentration gradient (Kanner and Bendahan, 1982). The uptake of one glutamate is coupled to the cotransport of three Na$^+$ ions and one H$^+$ ion and the counter transport of one K$^+$ ion (Zerangue and Kavanaugh, 1996). How the thermodynamically coupled transport of glutamate, H$^+$, Na$^+$, and K$^+$ ions is accomplished by glutamate transporters is not known. Here we present evidence for a binding site for Na$^+$ ions that suggests a mechanism for the coupling of sodium and glutamate transport.

At least one extracellular Na$^+$ ion appears to bind before glutamate can bind, and at least one extracellular Na$^+$ ion appears to bind after glutamate has bound to the transporter (Larsson et al., 2004; Wadiche et al., 1995b; Watzke et al., 2001). For example, in the absence of glutamate, a fluorophore attached to a cysteine at position A430 on HP2 in EAAT3 reported voltage- and Na$^{+}$-dependent fluorescence changes, consistent with Na$^+$ binding to the glutamate-free transporter and inducing a conformational change in HP2. Li$^+$ also supports glutamate uptake in the EAAT3 subtype, but with lower affinity than Na$^+$ (Borre and Kanner, 2001; Larsson et al., 2004). In addition, the affinity for different transported amino acids was shown to depend on the nature of the cotransported cation.
For example, the glutamate affinity was 130-fold less in Li⁺ than in Na⁺, whereas the aspartate affinity was only 10-fold less in Li⁺ than in Na⁺ (Menaker et al., 2006). The molecular mechanism for this cation specificity of the amino acid affinity is not known.

Mutagenesis experiments have identified residues affecting Na⁺ affinity and/or Li⁺/Na⁺ selectivity of glutamate transporters. For example, in EAAT3 the mutation T370S in TM7 lowered the affinity for Na⁺ and abolished Li⁺-driven glutamate uptake (Borre and Kanner, 2001). D368N in TM7 lowered the affinity for Na⁺ binding to the glutamate-free transporter (Tao et al., 2006). In the crystal structure of the bacterial glutamate transporter homologue GltPh (Yernool et al., 2004), the side chains of the homologous residues of D368 and T370 are far apart (Fig. 2.1A). These residues have been suggested to be part of different Na⁺-binding sites in EAAT3 (Yernool et al., 2004).

In a high-resolution crystal structure of GltPh (Boudker et al., 2007), two thallium cations were seen bound to the transporter. These cations were assumed to be binding to two of the Na⁺ sites, which were called “Na1” and “Na2” (Fig. 2.1). Na1 was localized close to D455 (EAAT3 numbering) in TM8 (Boudker et al., 2007). Na2 was localized at the tip of HP2 and at break of the alpha helical region in the middle of TM7 (Boudker et al., 2007) and was not located close to any of the residues identified by mutagenesis that affected Na⁺ binding (Fig. 1). The Na2 site was proposed to be the Na⁺ site that is occupied after glutamate has bound to the transporter (Boudker et al., 2007).

Using voltage clamp fluorometry (VCF), we sought here to determine the effects of different mutations on Na⁺ binding to the glutamate-free transporter. To guide our experimental studies, Grand-Canonical Monte-Carlo (GCMC) and molecular dynamics
(MD) simulations on Glt_{Ph} and a homology model of EAAT3 were performed to identify plausible locations of the ion binding sites. MD and free energy perturbation (FEP) simulations were used to assess the stability of the identified ion-binding sites. The experimental data and the computer simulations suggest that bound amino acid substrate and the side chains of T370 and N451 bind the third Na\(^+\) ion essential for the transport to occur.

2.3 Results

2.3.1 Computations suggest a Na site close to T370

We first try to locate potential third Na\(^+\) site using simulations based on the crystal structures of the bacterial EAAT3 homologue Glt_{Ph} and homology-modeled EAAT3 (see Methods in Supplementary Materials). In contrast to the moderate overall sequence identity (<40 %) between Glt_{Ph} and EAAT3, several functional regions are highly conserved throughout the family (Yernool et al., 2004). For example, the binding pocket for cations and bound amino acid substrate has at least 65 % homology. Therefore, we replaced amino acid L328 to D466 of Glt_{Ph} with the corresponding amino acids of EAAT3, creating a Glt_{Ph}-EAAT3 chimera. Three Na\(^+\) ions are cotransported with one glutamate in both the mammalian EAATs and bacterial Glt_{Ph} (Groeneveld and Slotboom, 2010; Zerangue and Kavanaugh, 1996). Known crystal structures of Glt_{Ph} have revealed only two putative sodium-binding sites (Boudker et al., 2007). We used probe particle insertion into Glt_{Ph} in GCMC simulations (Woo et al., 2004) to uncover possible locations of the third ion binding site. Methods and controls regarding stability and selectivity of binding pockets are summarized in Supplementary Materials.
2.3.2 GCMC/MD simulations of Glt\textsubscript{PH}

GCMC/MD insertions were run on two different isoforms of Glt\textsubscript{PH} (with either an open or closed extracellular gate, henceforth called the “open” and “closed” isoforms) with either no bound Na\textsuperscript{+} (‘ion free’) or with two Na\textsuperscript{+} ions bound to the two earlier proposed Na1 and Na2 sites (‘ion loaded’). We evaluated the electrostatic component of the binding free energy as well as Li\textsuperscript{+}/Na\textsuperscript{+} selectivity of the proposed ion binding sites. GCMC/MD simulations of the ion-free closed Glt\textsubscript{PH} isoform identified two sites similar to the crystallographic sites Na1 and Na2 plus three potential binding sites for a third Na\textsuperscript{+} ion dubbed “Na3”, “Na3’”, and “Na3’” (Fig. 2.1, for Na3’ and Na3’’ see Fig. S2 in Supplementary Materials section of the published paper). Further MD simulation shows that a Na\textsuperscript{+} ion at Na3 is stably bound at this site for more than 10 ns, whereas coordination at sites Na3” and Na3’ is significantly altered in the transporter with three bound ions. However, a Na\textsuperscript{+} at Na3’ is stable in the absence of a Na\textsuperscript{+} ion at Na1, and we therefore call Na3’ a “transient initial sodium-binding site” (Discussion). Na3’ is formed by ligands from D312, N401 and N310 residues. The combination of all simulation methods places the third Na\textsuperscript{+} ion (Na3) close to the side chain of T314 and the backbone oxygen of A353, with possible involvement of N401 side-chain oxygens atoms (homologous to T370, A409, and N451 in EAAT3, respectively) and in direct contact with the charged β-carboxylate group of the bound substrate (Fig. 2.1). After 10 ns of MD run, the average root mean square deviation (RMSD) of the heavy backbone atoms of the transporter relative to the initial structure was 2.7, 2.6 and 2.9 Å, for an Na\textsuperscript{+}-free structure (2nwl), a two-ion structure (2nwx; Na1-Na2, Fig. S5, Supplementary Materials section of the published paper) and our new structure with the third Na\textsuperscript{+} bound (Na1-
Na2-Na3; Fig. 2.1, and Fig. S3, Supplementary Materials section of the published paper). The average distances from this potential site Na3 to sites Na1 and Na2 are 6.9 and 10.8 Å, respectively. The combination of results from equilibrium MD and FEP data on the ion uncharging in this binding site suggests that this position for the Na3 site might be stable.

Figure 2.1. Putative Na⁺ binding sites in glutamate transporters. A) Model with 3 Na⁺ sites (Na1-Na2-Na3) suggested by the Grand-Canonical Monte-Carlo Simulations. Residues are numbered as in EAAT3. Two views of the C-terminal half of one subunit, rotated 90 degrees. HP1: yellow, HP2:green, TM7:purple, TM8:blue. Na⁺ are shown as yellow balls. B) The Na⁺ at Na3 interacts with the side chains of T370 and N451 and with the backbone oxygen of A409 (corresponding to T314, N401, and A353 in Gltph) and is in direct contact with the bound substrate. For more details see Figs. S3-S4 and Tables S1-S3 in Supplementary Materials section of the published paper.

2.3.3 GCMC/MD simulations of EAAT3

GCMC/MD insertions were also run on ion-free closed and open isoforms of the GltPh-EAAT3 chimera. The results of GCMC simulation for the chimera with the EAAT3 binding pocket are slightly different from the results for GltPh. In the closed isoform of the chimera, only three binding sites were identified initially, and all these binding sites were proven stable in 10-ns MD simulation performed on EAAT3 chimera. Site Na1 is formed by three carbonyl oxygen atoms from G362, N366 and N451 as well as by two
carboxylates from D455. Site Na2 is formed by carbonyl oxygen atoms from T364, I365, S405, I406 and A408. Site Na3 is formed by side-chains of T370 and N451, as well as by oxygens from the bound glutamate. The distance between the three bound sodium ions is around 7.1-7.5 Å, indicating an almost ideal triangular arrangement in EAAT3 after MD simulation (Fig. S3, Supplementary Materials section of the published paper). The stability of the model is encouraging but is insufficient to justify use of this model for further functional studies. This model illustrates similarities in ion/substrate binding in bacterial and mammalian transport systems. Therefore, all ion selectivity, substrate-binding, and ion-binding computations requiring a high-resolution structure were done on the bacterial GltPh.

2.3.4 Mutational studies suggest that T370 binds sodium before glutamate has bound

To test experimentally whether cations bind to the proposed Na3 binding site, we used VCF. To attach a fluorescent probe, we introduced a cysteine at position A430 in EAAT3 glutamate transporters. A430C transporters were expressed in Xenopus oocytes and labeled with the thiol-specific Alexa546-maleimide fluorophore. Using VCF, we have shown previously that the fluorescence from Alexa546-labeled A430C transporters reports on a conformational change induced by Na⁺ binding to the glutamate-free transporter (Larsson et al., 2004). Conformational changes induced by Na⁺ binding most likely alter the environment around the fluorophore, thereby causing changes in fluorescence. Fluorescence traces from A430C in 100 mM NaCl or 100 mM LiCl are shown in Fig. 2.2A. As reported earlier, fluorescent-labeled A430C displays voltage-dependent fluorescence changes both in Na⁺ and Li⁺ solutions, suggesting that both Na⁺
and Li\textsuperscript{+} binding induce conformational changes in HP2 (Larsson et al., 2004). The slopes of the fluorescence versus voltage curve F(V) are similar in Na\textsuperscript{+} and Li\textsuperscript{+} [Na\textsuperscript{+}: \(z\delta = 0.41 \pm 0.01\) (n=22), Li\textsuperscript{+}: \(z\delta = 0.36 \pm 0.01\) (n = 4)]. Na\textsuperscript{+} and Li\textsuperscript{+} ions thus bind at closely similar electrical distances into the membrane. (Fig. 2.2B). However, the F(V) in Li\textsuperscript{+} solutions is left-shifted by >50mV relative to the F(V) in Na\textsuperscript{+} solutions. We showed earlier that lowering the Na\textsuperscript{+} concentration 5 fold shifted the F(V) curve about the same amount (Larsson et al., 2004), as if Li\textsuperscript{+} has a 5-fold lower affinity than Na\textsuperscript{+}. In addition, the maximal amplitude of the fluorescence changes is smaller in Li\textsuperscript{+} compared to Na\textsuperscript{+} (Li\textsuperscript{+}: 3.08\% \pm 0.5\% \Delta F/F; Na\textsuperscript{+}: 5.36\% \pm 0.8\% \Delta F/F, n = 10), as if Li\textsuperscript{+} induces a smaller conformational change than Na\textsuperscript{+} or as if the efficacy of Li\textsuperscript{+} to induce conformational change is less than that of Na\textsuperscript{+}.

We next studied the mutation T370S in the background of A430C, to test to what extent the T370S mutation affects the interaction of Na\textsuperscript{+} with the glutamate-free transporter. Similar to what was reported earlier for T370S (Borre and Kanner, 2001), Alexa546-labeled T370S/A430C transporters conduct glutamate-induced transport currents in Na\textsuperscript{+}, but not in Li\textsuperscript{+} (Fig. 2.2C). We here show that in glutamate-free solutions Alexa546-labeled T370S/A430C transporters generate fluorescence changes in Na\textsuperscript{+}, but not in Li\textsuperscript{+} (Fig. 2.2D). The fluorescence changes in Na\textsuperscript{+} are similar to the fluorescence changes in 430C. However, the F(V) in Na\textsuperscript{+} for T370S/A430C is shifted to more hyperpolarized potentials (\(V_{1/2} = -60.9 \pm 1.5\) mV, (n = 6)), in the same direction as the F(V) for A430C in Li\textsuperscript{+} (Fig. 2.2E). Increasing the Na\textsuperscript{+} concentration from 100 mM to 200 mM for T370S/A430C shifts the \(V_{1/2}\) (\(V_{1/2} = -25.4 \pm 5.4\) mV, n = 4) towards the \(V_{1/2}\) of A430C. The apparent \(K_m\) for Na\textsuperscript{+}, measured from the amplitude of the Na\textsuperscript{+}-induced
fluorescence changes in different Na\(^+\) concentrations, increases at all voltages (Fig. 2.2F). For example at -80 mV, the Km is 61.7 ± 15 mM for 430C and 268 ± 41 mM (n = 4) for the T370S/A430C mutation.

Figure 2.2 T370S decrease the affinity for Na\(^+\). A&D) Fluorescence change in Na\(^+\) and Li\(^+\) for Alexa546-labeled A430C (A) and T370S/A430C (D) transporters in response to voltage steps from -200 mV to +200 mV in glutamate-free solutions. B&E) Steady-state F(V)s in Na\(^+\) (black) and Li\(^+\) (red) from A and D (measured at the arrows in A). The F(V)s were normalized to -1 at -200 mV in Na\(^+\). The data was fit with a Boltzmann curve: \( F = F_0/(1+\exp(-z\delta(V-V_{1/2})/kT)) \). In B, dashed line shows the fit of the Li\(^+\) data normalized to -1. In E, dashed line shows the fit of the Li\(^+\) (red) and Na\(^+\) (black) data for A430C from B. C) Glutamate-activated I/V curves in Na\(^+\) (black) and Li\(^+\) (red) for Alexa546-labeled T370S/A430C transporters. F) Voltage dependence of the Km for Na\(^+\) for A430C (black) and T370S/A430C (red). The Na\(^+\) dependence of the fluorescence for A430C and T370S/A430C was measured at different voltages and fit with the equation \( F(Na^+) = F_{\text{max}}/(1+Km/[Na^+]) \). Na\(^+\) was replaced by equimolar choline. G) Glutamate-activated I/V curves in Na\(^+\) for Alexa546-labeled T370A/A430C. H) Fluorescence in Na\(^+\) for Alexa546-labeled T370A/A430C in response to voltage steps from -200 mV to +200 mV in glutamate-free solutions.

We next tested the mutations T370A, which abolishes Na-driven glutamate uptake in EAAT3 (Borre and Kanner, 2001). Alexa546-labeled T370A/A430C transporters display no detectable glutamate-activated currents (Fig. 2.2G) or Na\(^+\)-

dependent fluorescence changes (Fig. 2.2H). Other T370 mutations, such as T370R displays no detectable glutamate-activated currents or Na\(^+\)-dependent fluorescence changes (Fig. 2.5).

A simple interpretation of these observations is that T370 forms part of a Na\(^+\) binding site in the glutamate-free transporter and that the conservative mutation T370S retaining a hydroxyl group still allows cation binding (but with a lower affinity and different selectivity), whereas less conservative mutations abolish Na\(^+\) binding. The fact that Li\(^+\) does not induce fluorescence changes in the T370S mutations suggests that T370S also alters the cation selectivity of this cation binding site, as is expected if T370 forms part of a cation binding site in EAAT3.

**2.3.5 Mutational studies suggest that N451 binds sodium before glutamate has bound**

Our computer simulations suggested that residue N451 contributes to the Na3 site in EAAT3 (Fig. 2.1) and plays an important, if not critical, role in the formation of the transient binding site Na3' in the open transporter. We therefore tested mutations of N451. Alexa546-labeled N451Q/A430C and N451A/A430C transporters display no detectable glutamate-activated currents (Fig. 2.3C) or radioactive glutamate uptake (Fig. S6, Supplementary Material). Alexa546-labeled N451Q/A430C transporters generate fluorescence changes in Na\(^+\) and Li\(^+\) (Fig. 2.3A). The F(V)s were shifted to more hyperpolarized potentials (Na\(^+\) :V\(_{1/2}\) = -97.0 ± 1.5 mV, (n = 12); Li\(^+\) :V\(_{1/2}\) = -156.9 ± 1.5 mV, (n = 9)) compared to the F(V) for 430C (Fig. 2.3B), as if the N451Q mutation lowers the affinity for both Na\(^+\) and Li\(^+\). In contrast, Alexa546-labeled N451A/A430C transporters display no Na\(^+\)-dependent fluorescence changes (Fig. 2.3D). These data are
consistent with our computer simulations suggesting that both T370 and N451 contribute to the Na3 site in EAAT3. This suggests that binding of Na+ to both T370 and N451 is necessary for inducing the conformational change reported by a fluorophore attached at 430C.

2.3.6 Mutational studies suggest that D368 binds sodium before glutamate has bound

We next tested the mutation D368N, which significantly lower the cation affinity of the glutamate-free transporter (Tao et al., 2006). Alexa546-labeled D368N/A430C transporters display no detectable glutamate-activated currents (Fig. 2.3E) or Na+-dependent fluorescence changes (Fig. 2.3F). Oocytes injected with T370A/A430C, N451A/A430C, or D368N/A430C are highly labeled with Alexa488-maleimide compared to uninjected oocytes (Fig. 2.3G). The amount of fluorescence labeling for these mutations were similar to that of the A430C mutation, showing that the absence of Na+-dependent fluorescence changes in these mutations were not due to a decreased expression in the cell membrane. These fluorescence data, showing that either mutation T370A, N451A, or D368N abolish the Na+-dependent fluorescence change, are consistent with our computer simulations suggesting that T370, N451, and D368 contribute to the Na3 (or Na3') site in EAAT3. This suggests D368 is necessary for inducing the conformational change reported by a fluorophore attached at 430C. Our simulations suggest that D368 site (Na3') plays an important role attracting sodium ion in the absence of bound glutamate. However, our simulations show that simultaneous occupancy of both Na1 and Na3' at D368 is prohibited due to very strong electrostatic
repulsion between ions separated by less than 3.5 Å (Fig. S4B, Supplementary Materials section of the published paper).

**Figure 2.3 T370A and D368N eliminate Na\(^{+}\)-dependent fluorescence changes.** A) Fluorescence change in Na\(^{+}\) and Li\(^{+}\) for Alexa546-labeled N451Q/A430C transporters in response to voltage steps from -200 mV to +160 mV in glutamate-free solutions. B) Steady-state F(V)s in Na\(^{+}\) (black) and Li\(^{+}\) (red) from A. C&E) Glutamate-activated I/V curves in Na\(^{+}\) for N451Q/A430C (C) and D368N/A430C (E). D&F) Fluorescence in Na\(^{+}\) for Alexa546-labeled N451Q/A430C (D) and D368N/A430C (F) in response to voltage steps from -200 mV to +160 mV in glutamate-free solutions. G) Alexa546-maleimide fluorescence labeling for oocytes expressing A430C, T370S/A430C, T370A/A430C, D368N/A430C, N451Q/A430C, and N451A/430C. The fluorescence was normalized to the fluorescence of uninjected (UI) oocytes.

**2.3.7 Functional role of the third binding site assessed by computations**

Our simulations show that a transporter with only 2 Na\(^{+}\) bound is unable to bind amino acid substrate favorably (Table 2.1). The introduction of the third ion in direct contact with the co-transported substrate leads to dramatic changes in the binding affinity, stabilizing the amino acid substrate in the binding pocket (Table 2.1). The removal of the positive charge from direct contact with the substrate (Na1:Na2 occupied state) led to unfavorable binding of the negatively charged aspartate. To further assess the cation binding to the proposed binding sites in Glt\(_{PH}\) we compute the free energy of ion binding to the different Na\(^{+}\) binding sites using FEP. We compute the electrostatic component of the free energy of ion binding for both the Na1-Na2 model and Na1-Na2-Na3 model to
illustrate the effect of the presence of the third ion on the absolute binding affinity of the other sites (Na1 or Na2). The electrostatic components of the affinities for Na$^+$ are -17.2 and -4.9 kcal/mol for Na1 and Na2, respectively, in the two-ion Na1-Na2 model simulations in the presence of aspartate. The computed binding affinities for Na$^+$ are -12.5, -4.7, and -10.1 kcal/mol for Na1, Na2, and Na3, respectively, in the three-ion Na1-Na2-Na3 model simulations in the presence of aspartate.

The ion binding affinity to Glt$_{Ph}$ is drastically different between the sites Na1 and Na2. Na1 is a high-affinity binding site for Na$^+$, whereas site Na2 displays only marginally favorable affinity for Na$^+$. The predicted third site (Na3) has relatively high affinity for Na$^+$ as evaluated by the absolute binding free energy from FEP charging/uncharging simulations. As expected, the presence of an ion at site Na3 leads to a small decrease in binding affinity for site Na2. This result is entirely expected, since two ions residing in sites Na2 and Na3 are separated by only about 8 Å, and unfavorable electrostatic repulsion between the two ions will affect ion-binding affinity at these sites. This combination of low-affinity and high-affinity ion binding sites is also similar to that of leucine transporter LeuT, suggesting different functionality of these two sites (Caplan et al., 2008).
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<td>∆Gdisp</td>
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<td>∆Grep</td>
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Table 2.1. Absolute free energy of binding (ΔG_{tot}) for aspartate to the reduced (GSBP) Na1-Na2 and Na1-Na2-Na3 GltPH structure. The absolute binding free energy (ΔG_{tot}) was decomposed into several contributions via staged protocol, namely electrostatic component (ΔG_{elec}), dispersive and repulsive parts of van der Waals interactions (ΔG_{disp} and ΔG_{rep}), configurational constraint component (ΔG_{const}), and bias from rotational and translational constraining potentials. Units are kcal/mole..

2.4 Discussion

Our results lead us to suggest the following model for extracellular Na\(^+\) and glutamate binding to glutamate transporters (Fig. 2.4).

i) There are two cation binding sites in glutamate-free transporters: Na1 contributed by D455 and Na3 contributed by T370 and N451 and transiently by D368. Equilibrium MD simulations suggest that D368 coordinates an early Na\(^+\) binding site (Na3') to the ion- and glutamate-free transporter. However, upon Na\(^+\) binding to Na1, the Na\(^+\) at Na3' quickly (<1 ns) moves over to the Na3 site with T370 (similar results were obtained for D312/T314 sites in the GltPH transporter) due to electrostatic repulsion between ions at the Na1 and Na3' sites. We therefore conclude that Na\(^+\) at Na3' site is only a transient Na\(^+\)
site, and that, once the transporter is fully occupied, the three Na\(^+\) will bind to Na1, Na2, and Na3 sites. The first two of the 3 coupled Na\(^+\) bind to these Na1 and Na3 sites in glutamate-free transporters. It should be noted however, that D312 may coordinate Na1 as it was found from equilibrium MD simulations (see Fig. S4 Supplementary Materials).

ii) The presence of a bound Na\(^+\) ion at Na3 induces a conformational change of HP2 that creates the binding site for glutamate. This is the conformational changes reported by the fluorophore in Figures 2.2-2.3. The Na\(^+\) bound at Na3 forms part of the glutamate binding site.

iii) Glutamate binding induces a conformational change that closes HP2 around the bound glutamate, which brings HP2 and TM7 close together as seen in the Glt\(_{Ph}\) crystal structure (Boudker et al., 2007).

iv) The glutamate-induced movement of HP2 creates the binding site for the last Na\(^+\) (Na2), which is found between TM7 and HP2 (Fig. 2.1A) in the crystal structure (Boudker et al., 2007).

Experimentally mutations of T370 (T314) and N451 (N401) altered the ability of Na\(^+\) to induce conformational changes in the absence of glutamate and mutations of T370 also changed the cation selectivity for these conformational changes, consistent with our computer simulations that suggested that both of these two residues contribute to our new Na3 sites in glutamate-free EAAT3 (Fig. 2.1B). Mutations of D368 also prevented Na\(^+\) to induce the conformational changes underlying the fluorescence changes in the absence of glutamate, in accordance with earlier studies showing that D368N altered the affinity for Na\(^+\)-induced leak currents in the glutamate-free transporter (Tao et al., 2006). In addition,
our simulations suggest that the Na\(^+\) bound at T370 interacts directly with the bound amino acid and affects the affinity of the bound amino acid substrate.

Earlier theoretical studies have also suggested that the third Na\(^+\) binds in the proximity to the bound amino acid substrate (Holley and Kavanaugh, 2009; Shrivastava et al., 2008). Kavanaugh and colleagues using the knowledge-based method VALE identified a putative third ion binding site, in which Na\(^+\) was coordinated by the bound amino acid substrate and three backbone oxygens from HP2 (Holley and Kavanaugh, 2009). T370 was not predicted to form part of this Na\(^+\) site (Holley and Kavanaugh, 2009). Shrivastava and coworkers noted that T370 (T314 in Glt\(_{Pb}\)) is able to bind water and that this water, in combination with the bound glutamate, could participate in coordinating a Na\(^+\) (Shrivastava et al., 2008). Taken together, both thermodynamic and steric considerations point towards the potential location of an ion binding site in this region, in good agreement with our results from GCMC/MD modeling.

The novel site (Na3), formed by the side chains of T370 and N451 and the backbone oxygen of A409, is reminiscent of that found in another Na\(^+\)-coupled transporter LeuT (Fig. 1) (Yamashita et al., 2005). One similar feature of this new site (Na3) in EAATs is that the cation interacts directly with the bound substrate, as was found for one cation in LeuT (Yamashita et al., 2005). Another similarity with LeuT is that there is a direct structural coupling from this cation site (Na3) to one of the other Na\(^+\) sites (Na1) in the original crystal structure, because the backbone of N451 forms part of the binding site for Na1. Our molecular dynamics simulations suggest that the Na\(^+\) bound at T370 interacts directly with the carboxyl oxygen of the transported amino acid, such that the amino acid binding is coordinated by the Na\(^+\) at site Na3 (Fig. 2.1). Previously, it
has been shown that the affinity of the amino acid substrate depends critically on the type of co-transported cation (Menaker et al., 2006). For example, the affinity for L-glutamate is 130-fold lower in the presence of Li+ than in the presence of Na+, whereas the aspartate affinity is only 10-fold lower in Li+ than in Na+ (Menaker et al., 2006). Here, we found that the direct interaction between Na+ and transported amino acid are critical for favorable substrate binding to the transporter (Table 2.1). The difference in the binding affinity of aspartate between the two- and three-ions bound transporter is more than 20 kcal/mol, as evaluated with staged free energy simulations protocol developed previously (Zhao, 2010). The analysis of the path-dependent free energy decomposition into components suggests that the key reason for this favorable gain in binding energy is a change in the electrostatic component (Table 2.1). While in the binding pocket of the GltPh transporter, the negatively-charged aspartate is unable to compensate for the loss of the favorable electrostatic interaction with bulk water, amounting up to -140 kcal/mol (Banavali, 2002), without direct coupling to the positively-charged cation.

The direct contact of the cation at site Na3 to the transported amino acid could underlie the Na+/Li+ selectivity of the apparent affinity for the transported amino acid in EAAT3. Another possibility suggested by our fluorescence data (Fig. 2.2B) is that Li+ is less efficient than Na+ to induce the conformational change once it is bound to the transporter or that Li+ binding does not induce as large conformational change as Na+. This latter possibility would also explain why the affinity of cysteine – which lack the γ-carboxylate group of glutamate that would interact directly with the Li+ - also changes dramatically in Li+ compared to in Na+ (Menaker et al., 2006). We therefore propose that there is both a cation specific effect on the conformational change of HP2, as well as a
direct interaction of the bound cation with substrates that contain a β- or γ-carboxylate group, such as aspartate and glutamate. This intimate link between the cation and the amino acid substrate in the glutamate transporter is similar to the interaction shown in the crystal structure for the LeuT transporter (Yamashita et al., 2005), where the Na⁺ and the substrate are bound together, and suggest a simple mechanism for how the transport of Na⁺ and co-transported substrate are tightly coupled in glutamate transporters.

2.5 Materials and methods

**Molecular Biology.** Site-directed mutagenesis of the human EAAT3, in vitro synthesis of RNA, and RNA injection into *Xenopus laevis* oocytes were performed as described previously (Larsson et al., 2004).

**Electrophysiology.** Glutamate-induced currents were calculated by subtracting the current measured in glutamate-free Na⁺ Ringer’s solution from the two-electrode voltage-clamp current measured in Na⁺ Ringer’s solution containing 1mM glutamate as described previously (Larsson et al., 2004). For Li⁺ experiments, Na⁺ was replaced by Li⁺.

**Voltage Clamp Fluorometry.** VCF experiments were performed as described previously (Larsson et al., 2004). Oocytes were labeled for 60 min with 10 μM Alexa-546 maleimide (Molecular Probes) in Na⁺ Ringer’s solution. Fluorescence was monitored through a rhodamine filter cube (HQ545/x30, Q570LP and HQ620/60m), low-pass filtered at 200-500 Hz, and digitized at 1 kHz.

**Grand-Canonical Monte-Carlo Simulations.** For this computation, as well as for the free energy of binding, only a sphere of 15 Å around the bound substrate is treated explicitly and the rest of the system is accounted for via generalize-solvent boundary
potential developed earlier (Banavali, 2002). The GCMC simulation was run for 300 cycles, each with 20000 MC steps. The structures with inserted water molecules were relaxed for 50 ps of Langevin dynamics for every GCMC cycle. These two approaches combined together provide a comprehensive description of the electrostatic map and accessibility, thus helping in identification of buried metal sites in proteins. A similar methodology has been used by T. Beck’s group (Kuang et al., 2008).

**Molecular Dynamics Simulations.** MD simulations were run on optimized structures of Glt\textsubscript{Ph} using the CHARMM c33b2 (Brooks, 1983). Na\textsuperscript{+} ions were introduced at the binding pockets identified in GCMC calculations. The simulation box contained 1 Glt\textsubscript{Ph} transporter (trimer), bound Na\textsuperscript{+}, 3 co-transported substrates and 298 DPPC lipid molecules, solvated by a 100 mM NaCl aqueous salt solution, comprising a total of \~120000 atoms. The lipid bilayer was built as described previously (Noskov, 2004). All simulations were carried at constant pressure (1 Atm) and constant temperature with periodic boundary conditions (Feller et al., 1995). Electrostatic interactions were treated using a particle mesh Ewald (PME) algorithm (Essman, 1995). All systems were equilibrated for 1.5 ns and then subjected to 5-15 ns runs to test stability.

**Free Energy Simulations: Evaluation of Absolute Ion and Substrate Binding Affinities.** Free Energy Perturbation (FEP) simulations were carried out using the CHARMM PERT command for Glt\textsubscript{Ph} containing two and three Na\textsuperscript{+} ions. The ligand binding computations were performed with staged protocol of Deng et al (Deng and Roux, 2009). Similar scheme has been used previously for studies of substrate and antidepressant binding to the neurotransmitter transporter LeuT (Zhao, 2010). The absolute binding free energies for ions were evaluated from FEP experiments with
periodic boundary conditions using parameters developed early (Caplan et al., 2008). The WHAM (weighted histogram analysis) method was used to post-process the FEP data and evaluate ion binding affinities for the binding sites (Na1, Na2, and Na3). The free energy of Na+ uncharging ($G^{\text{bulk elec}}_{\text{elec}}$) in the bulk water was estimated to be -98.6 kcal/mol (27). The absolute binding affinity (electrostatic component) was calculated as $\Delta G_{\text{elec}} = G^{\text{site elec}}_{\text{elec}} - G^{\text{bulk elec}}_{\text{elec}}$.

### 2.6 Supplementary information

The N451Q and N451A mutations prevent glutamate uptake: The N451Q and N451A mutations did not show any glutamate activated currents (Fig. 2.3C), suggesting that these mutations disrupt glutamate uptake by EAAT3. To further test whether glutamate uptake is accomplished in these mutations, we measured uptake of radio-active glutamate. Neither N451Q/A430C- nor N451A/430C-expressing oocytes displayed glutamate uptake compared to 430C-expressing oocytes (Fig. 2.4). The level of glutamate uptake for N451Q/A430C- and N451A/430C-expressing oocytes was similar to background glutamate uptake in uninjected (UI) oocytes (Fig. 2.4). Previously, it has been shown that the mutations T370A and D368N also prevent glutamate uptake in oocytes (Borre and Kanner, 2001). In contrast, the T370S mutation did show uptake in Na+, but not in Li+ (Borre and Kanner, 2001). The mutation D455N showed only uptake by homo-exchange, whereas more drastic mutation did not show any glutamate uptake (Teichman et al., 2009). For D455N, the authors suggested that the Na+-binding and the Na+ hemicycle of the transport cycle is intact, but that the K+ hemicycle of the transport cycle is disrupted (Teichman et al., 2009). All these uptake experiments are consistent
with our conclusion that T370, N451, D455, and D368 play important roles in the Na$^+$ binding of EAAT3, which is necessary for glutamate uptake by EAAT3.

![Figure 2.4 No glutamate uptake by N451 mutations in radiotracer flux experiment.](image)

Figure 2.4 No glutamate uptake by N451 mutations in radiotracer flux experiment. Five oocytes were placed in 250 µl Ringer solution supplemented with 0.3 µCi of L-[14C] glutamate for 3 minutes at room temperature. Oocytes were washed 4 times, before adding 10% SDS. Radioactivity was determined in a liquid scintillation counter.

Additional mutations of T370 are not functional: The mutation T370A abolished both the glutamate-activated current and sodium-dependent fluorescence changes (Fig. 2.2H). We also made the mutations T370R and T370K. The mutation T370R abolished both the glutamate-activated current and sodium-dependent fluorescence changes (Fig. 2.5), as if T370R disrupts sodium binding. T370K/430C expressing oocytes were not labeled over control (uninjected) oocytes and did not generate any glutamate-activated current, as if T370K disrupts the surface expression of EAAT3.
<table>
<thead>
<tr>
<th>Site</th>
<th>Glt&lt;sub&gt;Ph&lt;/sub&gt;</th>
<th></th>
<th>EAAT3</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Closed</td>
<td>Open</td>
<td>Closed</td>
</tr>
<tr>
<td>Na1</td>
<td>D405 (O, Oδ1, Oδ2)</td>
<td>D405 (O, Oδ1, Oδ2)</td>
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<tr>
<td></td>
<td>N310 (O)</td>
<td>N310 (O)</td>
<td>G362</td>
</tr>
<tr>
<td></td>
<td>N401 (O)</td>
<td>S278 (Oγ)</td>
<td>N451</td>
</tr>
<tr>
<td></td>
<td>G306 (O)</td>
<td></td>
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</tr>
<tr>
<td>Na3’</td>
<td>R&lt;sub&gt;276&lt;/sub&gt;(O), D&lt;sub&gt;394&lt;/sub&gt;(Oδ1, Oδ2)</td>
<td>R&lt;sub&gt;276&lt;/sub&gt;(O), D&lt;sub&gt;394&lt;/sub&gt;(Oδ1, Oδ2)</td>
<td>S334 (Oγ)</td>
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<tr>
<td></td>
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<td>G410 (O)</td>
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<td></td>
<td>S278 (Oγ)</td>
<td></td>
<td>V411 (O)</td>
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<td></td>
<td></td>
<td></td>
<td>S334 (O)</td>
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<tr>
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<td>A307(O)</td>
<td>N/A</td>
<td>Main-Chains of:</td>
</tr>
<tr>
<td></td>
<td>S349 (O)</td>
<td></td>
<td>I365</td>
</tr>
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<td></td>
<td>T352 (O)</td>
<td></td>
<td>I406</td>
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<td></td>
<td>T352 (Oγ)</td>
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<td>N401(Oδ1)</td>
<td>N401(Oδ1)</td>
<td>N401(Oδ1)</td>
</tr>
</tbody>
</table>

**Table 2.2.** The insertion sites in open and closed forms of the Glt<sub>Ph</sub> and EAAT3 transporters. The cutoff for insertion probability was set to be <1 %. The highest insertion probability is for the site Na1 ~14 %, the lowest for site Na3 ~5 %. The residues critical for all insertions are shown in bold e.g. comprising more than 75 % of the found sites. The remaining coordinating ligand may change. Sites are ranked by the probabilities of insertion as evaluated by the distance matrix. Sites Na3 and Na3’ may share N401 side-chain. Closed/open conformation refers to the position of HP2, which has been proposed to function as the external gate.
Figure 2.5 No glutamate-activated current or Na\(^+\)-dependent fluorescence changes from T370R mutation. A) Glutamate-activated I/V curves in Na\(^+\) for T370R/A430C. B) Fluorescence from Alexa488-labeled T370R/430C transporters in response to voltage steps from -160 mV to +160 mV in glutamate-free solutions.
Chapter 3

Transition metal FRET reports short range conformation change upon substrate binding and transport in human glutamate transporter

3.1 Introductory remarks

Glutamate transporters (also termed excitatory amino acid transporters (EAATs)) return glutamate concentrations to low levels in the extracellular space of the synaptic cleft following glutamate exocytosis (Danbolt, 2001). These transporters maintain a 10,000-fold gradient of intracellular to extracellular glutamate by coupling uptake of one glutamate molecule to the co-transport of three Na⁺ ions and a proton, and the countertransport of a K⁺ ion (Kanner and Bendahan, 1982; Pines and Kanner, 1990). Malfunction of this reuptake mechanism is implicated in stroke (Danbolt, 2001). However, how these transporters accomplish this coupled transport is not completely clear. Our previous macroscopic FRET measurement on mammalian glutamate transporters suggested that only small-scale molecular motions accomplish glutamate uptake (Koch and Larsson, 2005). In contrast, crystal structures from the glutamate transporter bacterial homologue Gltph show large conformational rearrangements (≈ 20 Å) during glutamate translocation (Boudker et al., 2007; Reyes et al., 2009; Yernool et al., 2004). The reason for this controversy is unknown. One possibility is that the human glutamate transporter and its archaeal homologue function by different mechanisms. Another possibility is that the difference between inward and outward conformations is not detected in the previous FRET analysis with traditional FRET pairs. To solve the controversy above and to determine the nature of the conformational changes that are associated with substrate binding and transport under physiological and
pathological conditions, we use a transition metal FRET assay on EAAT3 transporters in live cells.

3.1.1 The structure of glutamate transporters

To date no crystal structure of human glutamate transporter is available. But the archaeal glutamate transporter homologue Gltph, which shares a high sequence identity in the transport domain with the human homologues, have been crystalized in several states. Specifically, available Gltph crystal structures represent an outward-facing with extracellular gate open, outward-facing occluded with both extracellular and intracellular gates closed, and inward-facing occluded structure with both extracellular and intracellular gates closed (Boudker et al., 2007; Reyes et al., 2009; Yernool et al., 2004), suggesting the transporter undergo a series of conformation changes coordinated by co-transported ions and substrate (Fig. 3.1).

![Figure 3.1. Crystal structures of GltPH with different conformations. A) Outward facing substrate-bound state. B) Outward facing L-TBOA-bound state. C) Inward facing substrate-bound state. (Boudker et al., 2007; Reyes et al., 2009; Yernool et al., 2004). Cartoon representation of a single protomer.](image)

The Gltph outward-facing with extracellular gate open crystal structure is acquired with L-threo-β-benzzyloxyaspartate (L-TBOA), a non-transportable substrate analogue. In this structure L-TBOA locks the transporter in an outward-facing conformation similar to the aspartate-bound outward-facing occlude complex, except that hairpin 2 (HP2) adopts
an “open” conformation (Boudker et al., 2007; Shimamoto et al., 1998). In the TBOA-bound structure, HP2 moves ~10Å away from the substrate binding site, exposing the substrate binding site to the extracellular solution. A recent electron paramagnetic resonance (EPR) study in Gltph further showed opposite HP2 movements upon binding Na⁺ compared with substrate (Focke et al., 2011). Therefore, HP2 is proposed to serve as the extracellular gate of the glutamate transporter.

The mechanism of ion/substrate translocation across cell membrane is suggested by the Glpht inward-facing occluded structure (Reyes et al., 2009). Compared to the Gltph outward-facing structures, the transport domain containing substrate binding sites moves approximately 18 Å from near the extracellular solution to near the cytoplasm, accompanied by large conformational rearrangements of the transporter structure. This large structural change between extracellular (outward)-facing and intracellular (inward)-facing is further confirmed by recent double electron-electron resonance (DEER) spectroscopy and single-molecule FRET experiments (Akyuz et al., 2013; Erkens et al., 2013; Georgieva et al., 2013; Hanelt et al., 2013). Based on these findings the proposed model is that the binding of three Na⁺ ions and one glutamate closes the extracellular gate hairpin 2 (HP2), which initiates the translocation of the binding pocket across the membrane.

However, the conformational change related with steps of ion and substrate binding in mammalian glutamate transporters are largely unknown. The present models of EAATs are based on the crystal structures of Gltph (Fig. 3.2). To be noted, differences in mechanism of function between mammalian glutamate transporters and bacterial homologues could potentially exist because mammalian glutamate transporter require one
K⁺ ion in the countertransport step to complete the transport cycle (Fig. 3.2), while for Glp7 the empty transporter can isomerise between inward and outward-facing conformations without the assistance of K⁺. The functional differences between the two homologues prompted us to further investigate the transport mechanism of human glutamate transporters.

Previous studies on human glutamate transporter, such as voltage clamp fluorometry (VCF), suggested that there are significant Na⁺-dependent movements of the extracellular gate HP2 preceding glutamate binding (Larsson et al., 2004). Here with the transition metal FRET method, we further study what these HP2 conformations are under different ion and substrate conditions in human glutamate transporter EAAT3. In addition, with the transition metal FRET method, we look for the outward/inward-facing conformations of EAAT3 under different ion and substrate environments, to resolve the controversy of whether there is a large conformational change in the translocation step in the human glutamate transporter.

Figure 3.2. Hypothesis model for EAAT3 transport cycle. State 4) represents the crystal structure in Fig. 3.1.A and state 5 represents the crystal structure in Fig. 3.1.C. State 2 is thought to be similar to the L-TBOA bound structure in Fig. 3.1.B.
3.1.2 Transition metal fluorescence resonance energy transfer (FRET) to measure distances

FRET is a phenomenon by which a donor fluorophore, in its excited states, transfers its excitation energy to a nearby acceptor molecule (Lakowicz, 1999). The efficiency of FRET tapers off by the sixth power of the distance between the donor and acceptor molecule, making FRET ideal to measure small distance changes between different domains in proteins. The relative distance between FRET pairs can be estimated by measuring quenching of the donor fluorephore by an acceptor molecule. Recently, metal-ion FRET between a small fluorescent dye and a metal ion, like Ni$^{2+}$, bound to a dihistidine motif was developed to monitor small structural rearrangements in purified proteins (Taraska et al., 2009a; Taraska et al., 2009b). With fluorescein as FRET donor and transition metal ion Ni$^{2+}$ as FRET acceptor, $R_0$, the distance at which FRET efficiency is 50%, is 12 Å and could monitor short distance range between 5-25 Å (Taraska et al., 2009a), in contrast to most conventional FRET pairs which have $R_0$ of 30-60 Å and are thus not always suitable to measure intramolecular movements in proteins. In glutamate transporters, three identical subunits assemble into one transporter protein (Yernool et al., 2004). However, each subunit contains its own transport pathway and functions independently from other subunits (Koch and Larsson, 2005). Because transition metal FRET can work over shorter distances than classical FRET, it enables us to measure structural rearrangements within each subunit of the glutamate transporter without FRET interference from neighboring subunits. Here we use this method to measure the conformational changes of human glutamate transporter EAAT3 expressed in living cell membrane. By labeling the FRET pairs at different positions of EAAT3, we
can visualize the different conformational states of the EAAT3 transporter protein in different extracellular environments.

We find that hairpin 2 (HP2), the extracellular gate of human glutamate transporter is in the open conformation in the presence of competitive transporter blocker L-TBOA, corresponding well with the L-TBOA bound Glpth crystal structure. In contrast, HP2 is in a relatively closed conformation in Apo, Na\(^+\), K\(^+\) or Na\(^+\) and glutamate conditions, suggesting that the function of extracellular gate HP2 does not require as large an opening movement as sterically created by L-TBOA. EAAT3 labeled with FRET pair to investigate inward and outward states of glutamate transporters shows that there are large conformational changes between outward/inward states in human glutamate transporter. The transporters favor the outward-facing state in the presence of the extracellular competitive transporter blocker L-TBOA. They favor the inward-facing state in K\(^+\), while the outward-facing and inward-facing states distribution is in an intermediate range in Apo (ion and substrate free), Na\(^+\) or Na\(^+\) and glutamate. Interestingly, our FRET result suggests that both an “empty” and a “Na\(^+\) bound only” transporter in the absence of substrate glutamate can also isomerize between outward-facing and inward-facing conformations. By adding the extra “Empty isomerization” and “Na\(^+\) leak” steps to the transport cycle, we move a further step toward a complete understanding of the transport kinetics of glutamate transporter.
3.2 Results

3.2.1 Transition metal ion FRET can measure short distances in glutamate transporter proteins expressed in living cells

The transition metal ion FRET method we use here relies on the previous observation that the colored metal ion Ni\(^{2+}\) could be used as FRET acceptor for the fluorescent dye fluorescein. By engineering a metal ion-binding motif into \(\alpha\)-helices and by binding a fluorophore such as fluorescein onto an introduced cysteine residue, FRET could be used to observe small conformational rearrangements in proteins purified in detergent (Taraska et al., 2009a). The \(R_0\) value for Ni\(^{2+}\) and fluorescein is 12 Å and is suitable to monitor distance between 5-25 Å. FRET efficiency (E) could be easily determined by measuring donor fluorescein emission fluorescence before (F) and after (Fmetal) the addition of metals (E=1-Fmetal/F), and hence, the distance between the donor and acceptor could be determined.

Figure 3.3 Control FRET pairs labeling sites engineered into \(\alpha\)-helical transmembrane domain 2 (TM2). Glutamate transporter with di-histidine motif introduced at E57H/R61H on \(\alpha\)-helix TM2 for binding FRET acceptor Ni\(^{2+}\). Fluorescein-modified cysteine is introduced at A53C or K49C.
We here extend this technique to proteins expressed in living cells. To apply the transition metal FRET method to glutamate transporter protein EAAT3 expressed on *Xenopus* Oocyte, we first engineer the FRET pair labeling sites into the conserved α-helical transmembrane domain 2 (TM2), the rigid structure of the glutamate transporter that does not show position relocation according to different crystal structures of Gltph under different states, as a control. We select to mutate residues E57 and R61 (EAAT3 number) which are separated by one turn of the α-helix on TM2 into two histidines to form a metal ion binding site. We introduce the cysteine residue on A53 or K49 for the modification labeling of the reactive fluorophore fluorescein-5-maleimide (F5M) (Fig.3.3). All residues mutated for labeling of FRET pairs are accessible from the extracellular side of the transporter protein. EAAT3 protein constructs with introduced mutants are each expressed in *Xenopus* Oocytes.

**Figure 3.4** Average Ni\(^{2+}\) quenching of Fluorescein-5-maleimide reacted EAAT3 A53C, A53C/E57H/R61H or 49C/E57H/R61H. Oocytes expressing EAAT3 constructs are clamped at -30mV. Quenching experiment is performed in NaCl Ringer’s solution with increasing concentration of NiCl\(_2\). Fluorescence is normalized to the intensity before the addition of metal. FRET efficiency data is fitted from two-site binding model (solid lines)
We then perform fluorescence quenching experiments on EAAT3 control constructs with FRET pairs labeled on residues on one helix (TM2). In EAAT3 A53C/E57H/R61H with the metal binding site adjacent to the fluorophore, we observe substantial quenching of the emission fluorescence of fluorescein with increasing concentrations of Ni$^{2+}$. The $\beta$-carbon distance between the dihistidine motif and the engineered cysteine estimated from the crystal structures is closer in A53C/E57H/R61H than in 49C/57H/R61H. Correspondingly, the measured FRET efficiency decreases with distance in F5M-labeled EAAT3 constructs containing the dihistidine motif, for A53C/E57H/R61H 69% ± 1.5%; for 49C/57H/R61H 26% ± 1.0% (Fig. 3.4) (see calculation below).

The Ni$^{2+}$ dependent quenching of fluorescein emission fluorescence can be caused by 1) FRET from Ni$^{2+}$ bound at the engineered dihistidine motif, 2) FRET from Ni$^{2+}$ bound at native metal-ion-binding site in EAAT3 protein, 3) collisional quenching of fluorescein by Ni$^{2+}$ in solution. In F5M labeled EAAT3 construct with engineered cysteine but no engineered dihistidine motif, if fluorescein fluorescence is only quenched by nonspecific collisional quenching in solution, then the quenching curve can be fitted to a single-site binding curve model (see Methods), which could estimate the equilibrium dissociation constant for collisional quenching. However, if there is native metal-ion-binding site spatially close to the FRET donor fluorescein, FRET will occur between fluorescein and Ni$^{2+}$ bound at the native metal-ion-bindings site. The fluorescein quenching curve in Ni$^{2+}$ will show two stages of fluorescence decrease if the dissociation constant for the native metal-ion-binding site is different from collisional quenching. In this case a two-site binding curve model is used to estimate FRET efficiency from Ni$^{2+}$.
bound at the native metal-ion-binding site with nonspecific solution quenching of the fluorophore considered (see Methods). According to our data, in A53C without the engineered dihistidine motif, the Ni$^{2+}$ dependent fluorescein quenching curve shows two stages of fluorescence decrease and is best fitted by a two-site binding model (see methods) (Fig. 3.4), suggesting the EAAT3 protein contains a native metal-ion-binding site near A53C, with 15% ± 1.6% FRET efficiency and 300µM affinity. The distance between fluorophore labeled at A53C and the native Ni$^{2+}$ binding site is calculated as 16 Å (see methods). The equilibrium dissociation constant for solution quenching is measured as 40mM (Fig.3.4)

For EAAT3 A53C/E57H/R61H and 49C/E57H/R61H constructs, the data were fitted with two-site binding model (collisional quenching and FRET) (see methods) (Fig. 3.4). The collisional quenching dissociation factor is taken from the fitting of A53C (40mM) (Fig. 3.4). The measured FRET values are the combination of FRET to the native Ni$^{2+}$ binding site as well as the engineered sites. With an equation for FRET between one donor and multiple acceptors, we can calculate the distance to the engineered Ni$^{2+}$ binding motif, with FRET from native site corrected (see methods). In this way the total FRET measured in A53C/E57H/R61H (69% ± 1.5%) and 49C/E57H/R61H (26% ± 1.0%) is corrected with native site FRET measured in A53C (15% ± 1.6%). The measured distance between A53C and E57H/R61H is 11 Å; between K49C and E57HR61H is 16 Å. Oocytes expressing cysteine engineered EAAT3 transporters label significantly over background in uninjected oocytes labeled with FRET donor F5M. We correct for background fluorescence by subtracting the fluorescence of
F5M labeled uninjected oocytes in increased concentrations of Ni\(^{2+}\) from the fluorescence measured in the same batch of oocytes expressing EAAT3.

To ensure that the presence of Ni\(^{2+}\) does not interfere with Na\(^{+}\), glutamate and K\(^{+}\) binding to the glutamate transporter, we perform voltage clamp fluorometry on EAAT3 in the presence of Ni\(^{2+}\) (Fig. 3.5). Previously, by introducing a cysteine at position A430 in the EAAT3 transporter and subsequently labeling it with an environmentally sensitive fluorescent probe Alexa-546 maleimide, we showed that there is a different voltage dependent fluorescence change presumed to be due to conformational changes associated with l-glutamate, Na\(^{+}\) or K\(^{+}\) binding (Larsson et al., 2004). In the presence of 2mM Ni\(^{2+}\), a concentration of Ni\(^{2+}\) sufficient to saturate the engineered dihistidine motif according to fitted result from A53C/E57H/R61H (Kd=1.8*10\(^{-5}\)M), EAAT3 A430C labeled with fluorescein show similar VCF traces in the presence and absence of Ni\(^{2+}\) upon applied voltage protocol stimulation under different extracellular ion and substrate conditions.

The similar VCF traces in the presence or absence of Ni\(^{2+}\) suggest that the ion and substrate binding to the transporter is intact with Ni\(^{2+}\) around. Glutamate activated transport current of EAAT3 A430C/E57H/R61H is also intact with 2 mM Ni\(^{2+}\) in the extracellular solution. These data indicate that the transition metal ion Ni\(^{2+}\) and fluorescein pair can be used as a close-range distance-dependent tool to estimate distances in EAAT3 proteins expressed in *Xenopus* Oocyte.
Figure 3.5 Voltage- and substrate-dependence of A430C fluorescence. Representative fluorescence traces of fluorescein labeled EAAT3 A430C in response to voltage steps from -160mV to +160mV (increments of +20mV), from a holding potential of -30mV, in a single oocyte in Choline, NaCl, KCl Ringer’s solution before and after the presence of 2mM NiCl₂. The fluorescence trace of “Glu/NaCl” is measured in 200 µM glutamate in NaCl Ringer’s solution. The fluorescence trace of “Glu/NaCl + Ni²⁺” is measured in NaCl (100 mM) with 2 mM total glutamate (~200 µM free glutamate, see Fig. 3.7) with the presence of 2mM Ni²⁺.

3.2.2 The Extracellular gate HP2 adopts different conformations in competitive block L-TBOA and transportable ions/substrate

HP2 is suggested to serve as the extracellular gate in mammalian glutamate transporter. In the previously proposed transport cycle, the extracellular gate HP2 of an empty outward facing glutamate transporter is hypothesized to open first, exposing the substrate binding site for binding of substrate and ions, and then closes to trap the bound ion and substrate and initiate the subsequent translocation steps (Fig. 3.2). Here we aim to look into the conformational changes of HP2 upon ion, substrate or inhibitor binding. We
reason that the open and closed states of HP2 will result in distance changes between specific residues and that we could monitor these distance changes with transition metal FRET.

By examination of available Gltph crystal structures, we make two EAAT3 constructs V411C/E57H/R61H and I437C/Q413H/V417H (all residues are in conserved domains of glutamate transporters) which are expected to undergo 12-22 Å (V411C/E57H/R61H) or 6-12 Å (I437C/Q413H/V417H) distance changes between the aspartate-bound HP2 closed and L-TBOA-bound HP2 open structure (Fig. 3.6).

![Figure 3.6 Crystal Structure of Gltph with HP2 closed and open conformation. Distance between V411C and E57H/R61H changes from HP2 closed A) to open B). Distance between I437C and Q413H/V417H changes from HP2 closed C) to open D).](image)

Unlabeled EAAT3 V411C/E57H/R61H and I437C/Q413H/V417H generate glutamate-activated transport current, while the transport current is blocked by labeling
with FRET donor fluorophore fluorescein in both constructs. The elimination of glutamate transport by fluorescein labeling at V411C or I437C could be because, in the crystal structures of the inward-facing state of the transporter, these two residues are located within the transport domain and the attachment of a fluorophore molecule sterically would prevent the conformation change to the inward-facing state. Previous studies on mammalian transporters have shown that mutating and/or labeling of cysteines introduced in HP2 results in transporters that do not show significant uptake of radioactive substrates (Borre et al., 2002; Brocke et al., 2002; Ryan and Vandenberg, 2002; Seal et al., 2001). However, these studies also demonstrated that the mutating and/or labeling of cysteines introduced into HP2 does not prevent substrate binding or extracellular conformational changes in mammalian transporters. Furthermore, in F5M labeled V411C/E57H/R61H and I437C/E57H/R61H the voltage dependent fluorescence (VCF) change measured under different extracellular conditions shows that Na\(^{+}\), substrate and K\(^{+}\) could bind to the transporter and induce conformation changes (Fig. 3.5). This feature enables us to exclusively measure the HP2 conformation change related to Na\(^{+}\), glutamate and K\(^{+}\) binding from the extracellular side in outward-facing transporters, isolated from other states of the transporter cycle.

Because of the relatively high affinity between glutamate/L-TBOA and Ni\(^{2+}\) (Kd=5*10\(^{-6}\) M at PH=7.4), it is impossible to measure the fluorescein fluorescence quenching curve in multiple Ni\(^{2+}\) concentrations with glutamate or L-TBOA present without significantly altering the free glutamate or L-TBOA concentration. However, according to the Ni\(^{2+}\) dependent fluorescein quenching curve measured for V411C/E57H/R61H (Fig. 3.8), the affinity of E57H/R61H for Ni\(^{2+}\) is 17 \(\mu\)M. Thus at 100
µM to 2 mM Ni\(^{2+}\) concentration the dihistidine motif is saturated without much collisional quenching effect of free Ni\(^{2+}\) in the solution (as measured from quenching of A53C without dihistidine motif, where the collision quenching affinity is 40mM, Fig. 3.4). If we use 2 mM Ni\(^{2+}\) and 2 mM glutamate or L-TBOA total we expect to have around 100 µM free glutamate or L-TBOA and 100 µM free Ni\(^{2+}\) in the solution. We then further estimated the free glutamate concentration in the 2 mM glutamate and 2 mM Ni\(^{2+}\) solution with voltage clamp fluorometry (VCF). In Fig. 3.5 we showed that there is voltage depend fluorescence change in the presence of glutamate and NaCl for EAAT3 A430C labeled with fluorescein-5-maleimide. Further investigation shows that the voltage dependent fluorescence change (F/V) curves of EAAT3 A430C labeled with fluorescein-5-maleimide in NaCl Ringer’s solution with different concentrations of glutamate display a rightward shift with increasing concentrations of glutamate (Fig. 3.7).

![Figure 3.7 Glutamate concentration dependent steady-state F(V)s of fluorescein labeled A430C.](image)

Figure 3.7 Glutamate concentration dependent steady-state F(V)s of fluorescein labeled A430C. The voltage dependent fluorescence is measured in extracellular NaCl Ringer’s solution containing either 100 µM, 200 µM, 300 µM, 500 µM glutamate or 2 mM glutamate and 2 mM Ni\(^{2+}\).
We then compared the F/V measured in 2 mM total Ni\(^{2+}\) and 2 mM total glutamate with the F/V curves measured in different free glutamate concentrations on EAAT3 A430C labeled with fluorescein. The F/V curve measured in 2mM total Ni\(^{2+}\) and 2mM total glutamate followed most closely to the F/V measured in 200–300 µM glutamate without Ni\(^{2+}\) (Fig. 3.7), suggesting the free glutamate concentration in 2 mM total glutamate and 2 mM total Ni\(^{2+}\) solution is around 200 µM. Because Ni\(^{2+}\) and glutamate bind in a 1:1 fashion, the free Ni\(^{2+}\) concentration is also 200 µM. 200µm free glutamate or L-TBOA is enough to saturate the substrate binding site in the transporter and 200 µM free Ni\(^{2+}\) saturate the di-histidine motif for the FRET measurement.

To measure EAAT3 HP2 conformations in glutamate or L-TBOA, EAAT3 A53C/E57H/R61H, V411C/E57H/R61H and I437C/Q413H/V417H are each expressed in Xenopus Oocyte and labeled with fluorescein-5-maleimide. For each EAAT3 construct we first measure fluorescein emission fluorescence in response to voltage steps from -160mV to +160mV, from a holding potential of -30mV in 2 mM glutamate or 2mM L-TBOA (Bath solution: NaCl Ringer’s solution). Then, at the same excitation light intensity the same voltage protocol is run again in 2 mM glutamate or 2 mM L-TBOA in the presence of 2 mM NiCl\(_2\) (Bath solution: NaCl Ringer’s solution) and fluorescein emission fluorescence is recorded. Representative voltage clamp fluorometry recordings for each constructs is shown in Fig. 3.8. Background fluorescence is measured in F5M labeled uninjected oocytes and subtracted from the fluorescence measured in the same batch of oocytes expressing F5M labeled EAAT3. Based on the fluorescein emission intensity recorded at -30mV before and after the application of Ni\(^{2+}\), FRET efficiency is calculated. For both V411C/E57H/R61H and I437C/Q413H/V417H, FRET efficiency in
glutamate is significantly larger than in L-TBOA, indicating that the distance between FRET pairs is closer in glutamate than in TBOA for both constructs (Fig. 3.8.D). For V411C/E57H/R61H, the FRET efficiency in glutamate is 40% and in L-TBOA 26% (Fig. 3.8.B). The EAAT3 V411C Ni\(^{2+}\) quenching curve has 20% FRET from the native metal-ion-binding site (not shown), so we calculate the distance of V411C/E57H/R61H with the one donor and multiple acceptors equation (see methods). In glutamate the distance is measured as 14 Å and in L-TBOA 18 Å. For I437C/Q413H/V417H, FRET efficiency in glutamate is 47% and in L-TBOA 30% (Fig. 3.8.C). The distance of I437C/Q413H/V417H is not calculated because according to Ni\(^{2+}\) quenching curve of I437C/Q413H/V417H (Fig. 3.9.B), the affinity of Q413H/V417H for Ni\(^{2+}\) is relatively low (102μM). With 200μM free Ni\(^{2+}\), the dihistidine site is not well saturated in 2mM total glutamate/L-TBOA and 2mM total Ni\(^{2+}\) in the solution. Though we see a big FRET difference in I437C/Q413H/V417H in glutamate compared to in L-TBOA (Fig. 3.8.D), the distance cannot be accurately measured in this case. As expected, the control construct A53C/E57H/R61H, which is not expected to be altered by HP2 conformation rearrangement, does not show a significant FRET efficiency difference between glutamate and L-TBOA (Fig. 3.8.A). We interpret the long distance 18 Å measured in L-TBOA between E57H/R61H and V411C to represent L-TBOA bound HP2 open conformation of EAAT3, as in the GltpH crystal structure with L-TBOA bound. We interpret the short distance 14 Å measured in glutamate to represent HP2 in a relatively closed conformation compared to the open conformation in L-TBOA, corresponding well with the HP2-closed conformation in GltpH crystal structure with Na\(^+\) and aspartate bound.
Figure 3.8 Different EAAT3 HP2 conformations measured in glutamate and L-TBOA. A-C) Representative fluorescence in response to voltage steps from -160mV to +160mV (increments of +20mV), from a holding potential of -30mV, in a single oocyte in 2mM glutamate or 2mM L-TBOA before and after the application of 2mM NiCl₂ (Bath solution: NaCl Ringer’s solution) in A) A53C/E57H/R61H, B) V411C/E57H/R61H and C) I437C/Q413H/V417H. D) Averaged difference of FRET efficiency (E) in glutamate compared with in TBOA.
To study EAAT3 HP2 conformations in Apo (ion and substrate free), Na⁺ or K⁺, we measure quenching of the emission fluorescence of fluorescein with increasing concentrations of Ni²⁺ in V411C/E57H/R61H and I437C/413H/417H in the presence of choline (Apo), Na⁺ or K⁺. For both constructs, the measured FRET efficiency is similar in choline, Na⁺ or K⁺. The data is fitted with the two-site binding model for the Na⁺ data (see methods) (Fig. 3.9). FRET efficiency in NaCl is 40% ± 1.0% for V411C/E57H/R61H and 68% ± 2.3% for I437C/413H/417H. FRET from the native metal-ion-binding site for both V411C and I437C without the engineered dihistidine motif is ~20%, thus with the one FRET donor and multiple acceptor equation (see methods), we calculate the distance in NaCl between V411C and E57H/R61H as 14 Å, and between I437C and Q413H/V417H as 11 Å.

**Figure 3.9 Average Ni²⁺ quenching of Fluorescein-5-maleimide reacted EAAT3.** F5M fluorescence quenching in increasing concentration of NiCl₂ in NaCl, Choline or KCl Ringer’s solution is measured for A) V411C/E57H/R61H and B) I437C/Q413H/V417H. Na⁺ Data is fit by a two-site binding model (solid lines).

The 14 Å distance between V411C and E57H/R61H in NaCl, KCl and choline is the same as the distance measured in glutamate (14 Å) (Fig. 3.8). However, in L-TBOA
the distance is 18 Å. This result suggests that in EAAT3, HP2 adopts an extremely open conformation in the presence of the competitive blocker L-TBOA, probably because the extra ring of L-TBOA sterically pushes HP2 into the open conformation. However in Na⁺, K⁺, choline, or Na⁺ and glutamate, HP2 seems to have similar closed conformations, suggesting that the HP2 conformational changes during the binding of transportable ion and substrate do not necessarily require as large opening movement as in the presence of L-TBOA. Also due to the nature of the macroscopic static FRET measurement, the result represents averaged HP2 conformations of multiple glutamate transporters distributed into potentially different states. The averaged result will bias toward the most long-lived states. Thus it is also possible that HP2 can go through short-lived, transient “open” conformation similar to the HP2 open conformation in L-TBOA. However, HP2 is mainly in the closed conformation, even if HP2 could be very dynamic and flickers between open and closed conformations, in conditions as Apo, Na⁺, K⁺ or Na⁺ and glutamate.

3.2.3 FRET measurements of EAAT3 outward and inward-facing states

The translocation steps of the transport cycle in human glutamate transporters are the cotransport of bound glutamate, Na⁺ and H⁺ across the membrane and the countertransport of one K⁺ ion. According to the crystal structures from the bacterial glutamate transporter homologue Gltph, there are large-scale conformational changes between outward-facing and inward-facing conformations of glutamate transporter. Specifically, the transition between outward/inward states of the transporter is suggested to be accomplished by an ≈18Å movement of the substrate binding sites from near the extracellular solution to near the cytoplasm (Boudker et al., 2007; Reyes et al., 2009;
Yernool et al., 2004). This large scale movement was later confirmed by double electron-electron resonance (DEER) spectroscopy and single-molecule FRET measurements on Gltph (Akyuz et al., 2013; Erkens et al., 2013; Georgieva et al., 2013; Hanelt et al., 2013). The large conformational rearrangement between outward and inward-facing states is very surprising because we have previously used traditional FRET method to look into both inter-subunit and intra-subunit distance changes under different extracellular conditions in EAAT3 where no large-scale dynamic change was detected in different transport environments.

To resolve this discrepancy, we reason that one possible explanation is that in our previous FRET measurement, the condition (NaCl-Ringer solution with 1 mM glutamate) we used was not biasing the transport into the inward facing state as expected. Therefore, we first test different extracellular solutions that should bias EAAT3 into the outward or inward conformation, respectively, and repeat our earlier FRET experiment. We use Alex488 C5-maleimide as FRET donor and tetramethylrodamine-methanethiosulfonate as FRET acceptor as in our previous experiment (Koch and Larsson, 2005). To obtain the outward conformation of EAAT3, we tested NaCl or choline. To obtain the inward conformation of EAAT3, we tested KCl or NaCl with 1mM glutamate. However, similar to our published data, FRET measurements of intersubunit distances between A430C in EAAT3 still show very small distance changes (< 1Å) (Fig. 3.10.B), much smaller than the ~13Å distance change (16% change in FRET efficiency) predicted from the Gltph crystal structures (Fig. 3.10.A) (Boudker et al., 2007; Reyes et al., 2009; Yernool et al., 2004). In addition, no changes are seen in intersubunit FRET experiments on L387C (data not show), residues which are predicted to move closer by >20Å. Therefore, we
conclude that either different extracellular solutions cannot bias EAAT3 into different outward/inward conformations, or the FRET method with traditional FRET pairs in our precious experiment cannot effectively report the distance changes. Therefore a new strategy is needed to study the translocation step of mammalian glutamate transport.

Figure 3.10 No FRET efficiency change detected for A430C. A) Large change in intersubunit distance between A430C in Gltph crystal structure, shown from a top view of the trimer. B) FRET efficiency (E) measured differences between Na⁺ and K⁺ (Na-K), Na⁺ and Choline (Na-Ch), or Na⁺ and Na⁺/Glutamate (Na-Glu). To-Ti is the expected E change for the homologous residue in Gltph crystal structures between the outward- or inward-facing states.

Here we use the transition metal FRET to continue to investigate the outward/inward conformations of human glutamate transporter EAAT3 under different ion/substrate conditions. Based on Gltph crystal structures, we make A430C/E57H/R61H construct on the conserved region of EAAT3 for the labeling of transition metal FRET pairs F5M and Ni²⁺. The distance between A430 and E57H/R61C is ~28 Å in all available outward-facing Gltph crystal structures with different ligands bound (for example with L-TBOA or aspartate) while the distance is ~9 Å in inward-facing crystal structure, suggesting that the distance changes between these residues is the result of the translocation of the transporter between outward and inward states (Fig. 3.11.A) (Boudker et al., 2007; Reyes et al., 2009; Yernool et al., 2004). F5M labeled
EAAT3 A430C/E57H/R61H expressed in *Xenopus* Oocyte generate the same amount of transport current in the presence of 2 mM glutamate and 2 mM Ni$^{2+}$ as in the absence of Ni$^{2+}$, indicating the transporter functions normally and is able to translocate between outward and inward states after FRET pair labeling (Fig. 3.11.B).

**Figure 3.11 EAAT3 A430C/E57H/R61H to measure outward and inward conformations of glutamate transporter.** A) Distance between A430C and E57H/R61H change by 19 Å from outward-facing to inward-facing structure in Gltph. B) Glutamate activated transport current is intact in fluorescein labeled EAAT3 A430C/E57H/R61H in the presence of 2mM NiCl$_2$.

For FRET measurement EAAT3 A430C/E57H/R61H is expressed in *Xenopus* Oocyte and labeled with fluorescein-5-maileimide. We first measure fluorescein emission fluorescence in response to voltage steps from -160mV to +160mV, from a holding potential of -30mV in extracellular Choline, Na$^+$, K$^+$, Na$^+$ and glutamate, or Na$^+$ and L-TBOA. Then, at the same excitation light intensity the same voltage protocol is run again in all conditions above in the presence of 2mM NiCl$_2$ and fluorescein emission fluorescence is recorded. Representative voltage clamp fluorometry recordings for each condition measured in a single oocyte is shown in Fig. 3.12.A-E. Background fluorescence is measured in uninjected oocyte and subtracted from the fluorescence measured in the same batch of oocyte expressing EAAT3 A430C/E57H/R61H. Based on
averaged static fluorescein emission intensity recorded at each voltage step between -160mV and +160mV before and after the presence of Ni\(^{2+}\), FRET efficiency is calculated and then averaged between multiple cells (Fig. 3.12.F). The measured FRET efficiency in K\(^{+}\) is highest among all states tested, while in L-TBOA FRET is lowest. The FRET efficiency in Apo, Na\(^{+}\) and Na\(^{+}\)/glutamate is in the intermediate range (Fig. 3.12.F). At -20mV, FRET efficiency measured in 2mM Ni\(^{2+}\) in KCl is 44% ± 1.7%, in NaCl 26% ± 1.1%, in Choline 24% ± 1.3%, in NaCl and glutamate 30% ± 1.0%, in NaCl and L-TBOA 7.5% ± 2.1% (Fig. 3.12.F). Based on Ni\(^{2+}\) quenching curve of F5M labeled A430C without E57H/R61H (Fig. 3.12.G), the data is best fit with two-site-binding model (see methods) and the FRET from native Ni\(^{2+}\) ion binding site is measured as 20% ± 2.1% (Fig. 3.12.G). The affinity of the native metal ion binding site to Ni\(^{2+}\) is measured as 220 \(\mu\)M. Because of the relatively low affinity between the native metal ion binding site and Ni\(^{2+}\), in 2mM total glutamate or L-TBOA and 2 mM total Ni\(^{2+}\), the free Ni\(^{2+}\) in the solution (≈200 \(\mu\)M) can only saturate around 50% of the native metal ion binding site, therefore we estimate the FRET contributed from the native metal ion binding site in glutamate or L-TBOA conditions as 10%. In NaCl, KCl and choline conditions, the 2 mM free Ni\(^{2+}\) in the solution can mostly saturate the native metal ion binding site, therefore the FRET from native ion binding site is estimated as 20%. We then use the one donor multiple acceptor equation to calculate the distance between A430C and E57H/R61H in above conditions with the FRET from the native metal ion binding site corrected. At -20mV the distance in KCl is measured as 13 Å, in Glutamate/NaCl 15 Å, in NaCl 18 Å, in choline 20Å, in L-TBOA >25 Å.
Figure 3.12 EAAT3 A430C/E57H/R61H show different distributions between outward/inward states under different conditions. A-E) Voltage dependent fluorescence measured in a single oocyte expressing EAAT3 A430C/E57H/R61H in response to voltage steps from -160mV to +160mV (increments of +20mV) from a holding potential of -30mV, in L-TBOA, Choline, NaCl, glutamate or KCl before and after the application of 2mM NiCl₂. F) Average of voltage dependent FRET efficiency measured from multiple oocytes (n=5). G) FRET fluorescence quenching in increasing concentration of NiCl₂ in NaCl Ringer’s solution of A430C. Data is fit to a two-site-binding model (solid line).
Since the binding of the competitive transport inhibitor L-TBOA from the extracellular side to the transporter inhibits transport and prevents the translocation from outward-facing to inward-facing states, we interpret the lowest FRET efficiency (7.5% ± 2.1%) measured in L-TBOA to represent the transporters in the outward-facing state. The > 25 Å distance measured in L-TBOA between A430C and E57H/R61H corresponds very well with outward-facing Gltph crystal structures (28 Å) (Fig. 3.11.A). In K⁺, the measured FRET efficiency is highest among all conditions (44% ± 1.7%), representing largest ratio of transporters distributed in inward-facing states. High K⁺ at extracellular sites bias the transporters into inward-facing states through the countertransport cycle. The 13 Å distance measured in K⁺ is close to the prediction from inward-facing Gltph crystal structure (9 Å) (Fig. 3.11.A). Therefore, our FRET data show there is large conformation rearrangements between outward/inward states in human glutamate transporter. In Apo, Na⁺ only or Na⁺/glutamate conditions, the ratio of outward-facing and inward-facing transporter molecules are in the intermediate range, between Na⁺/L-TBOA and K⁺ conditions. This data suggest that besides isomerizing between outward/inward-facing states in Na⁺/glutamate bound or K⁺ bound states, as indicated from the transport cycle, interestingly, the transporter can also isomerize between outward/inward conformations in Apo (no ion/substrate bound) or Na⁺ bound only states. In addition, the measured FRET efficiency is voltage dependent in Na⁺/glutamate or K⁺ conditions. This result is expected because the cycle of glutamate transporter has several voltage dependent steps during ion/substrate binding and translocation (Fig. 3.13). Our FRET data show that for both the Na⁺/glutamate condition and the K⁺ condition, the transporter favors more inward-facing states at negative voltages than at positive voltages.
Based on previous studies on glutamate transporter, we incorporate our transition metal FRET results into a 16 state EAAT3 model, to simulate the full cycle of glutamate transporter. (see discussion, Fig. 3.13)

3.3 Discussion

In this study, we use transition metal FRET to study conformational changes in different ion/substrate conditions in the neuronal glutamate transporter EAAT3 expressed in *Xenopus* oocyte. According to our data, in a living cell environment with fluorescein-Ni$^{2+}$ FRET pair, the measured distance in EAAT3 constructs is overall in agreement with the $\beta$-carbon distance between corresponding residues in Gltph crystal structures, suggesting structural consistency between EAAT3 and Gltph homologues. In addition, our transition metal FRET measurement report change in relative distance between FRET pairs resulting from conformational rearrangements of the transporter protein upon ion/substrate binding and transport. These conformational changes in EAAT3 were not detected previously with traditional FRET pairs. In this study, the transition metal FRET pairs are more sensitive to report distance change than traditional FRET pairs, probably due to the smaller size and shorter linker of the transition metal FRET pair. In addition, the shorter $R_0$ of transition metal FRET pairs is better suited to monitor short-range distance within one subunit of the transporter protein, without complications from FRET pairs labeled in neighbor subunits.

Our measurement of the extracellular gate HP2 conformations in EAAT3 show that the competitive transporter blocker L-TBOA pushes HP2 into an “open” conformation, different from the more “closed” conformation in Na$^+$ or Na$^+$/glutamate or
K⁺ or Choline. Our data in L-TBOA is in agreement with L-TBOA bound outward-facing crystal structure with HP2 open. Interestingly, a recent EPR study on HP2 conformations in Gltph further shows that HP2 is in an “open” conformation not just in L-TBOA, but also in Na⁺, as if Na⁺ opens the extracellular gate to expose substrate binding sites; HP2 is in “closed” conformation in Na⁺/glutamate or choline, so the bound Na⁺ and glutamate could prepare to translocate across membrane, or the empty transporter (choline) could isomerize back to start the next transport cycle. However, here our FRET measurement in EAAT3 show that in Na⁺ or K⁺ or Choline, or Na⁺/glutamate, HP2 seems to have similar closed conformations. Our data suggest that in EAAT3, the HP2 conformational changes during the binding of transportable ion and substrate do not necessarily require as large opening movement as in the presence of L-TBOA. It is also possible that in EAAT3 HP2 is very dynamic and flickers between open and closed conformations in conditions as Apo, Na⁺, K⁺ or Na⁺ and glutamate. In this case due to the nature of our macroscopic FRET measurement, the result represents averaged HP2 conformations between open and closed conformations.

With transition metal FRET method, we detect large conformational changes (13 Å - 25 Å from K⁺ to L-TBOA, Fig. 3.12) representing outward/inward isomerizations in EAAT3. The large-scale conformation change is consistent with outward/inward Gltph crystal structures, suggesting that the structural translocation mechanism is similar between human glutamate transporters and their archaeal homologue. Recently, DEER spectrometry and smFRET were performed either in detergent or reconstituted proteoliposomes to study Gltph outward/inward conformational changes under different ion/substrate conditions (Akyuz et al., 2013; Erkens et al., 2013; Georgieva et al., 2013;
Hanelt et al., 2013). Their result suggest that the population distribution of outward/inward conformations could be shifted toward more outward-facing states or more inward-facing states by introduced mutants, ion gradient, different ion/substrate conditions, or changes from detergent to bilipid environment. But overall the transporter is always dynamic and isomerize between outward and inward conformations in all of Apo or Na$^+$ or Na$^+$/glutamate or Na$^+$/L-TBOA conditions. A previous cysteine accessibility study on the mammalian glutamate transporter Glt1 suggests that in K$^+$, the transporter spends more time in inward conformations than in Na$^+$ only, or in Na$^+$/glutamate. In contrast, in the presence of the extracellular transporter blocker kainate, the transporters are more locked in the outward conformations than in the Na$^+$ only condition (Shlaifer and Kanner, 2007), in excellent agreement with our transition metal FRET results in this study. To better understand the kinetics underlying the FRET efficiency changes, we modeled the FRET data by developing a 16 states kinetic model of EAAT3 based on our data and previous studies (Bergles et al., 2002; Larsson et al., 2004; Tzingounis et al., 2002) (Fig. 3.13).

In the model in Fig. 3.13.A, “$T_0$” stands for an empty EAAT3 transporter with nothing bound. Based on our FRET data the transporter still isomerize between outward and inward conformations in Apo (Choline) or NaCl conditions. So in this model we add two extra transitions between outward facing and inward facing states. One transition is between “$T_0$” and “$T_i$”, the other is between $T_0$Na$_2$H and $T_i$Na$_2$H. The rates and voltage dependence in our model are estimated based on previous EAAT3 models or taken from experimental studies (Bergles et al., 2002; Larsson et al., 2004; Tzingounis et al., 2002). With the two extra “empty isomerization” and “Na$^+$ leak” transitions added, our model
can reproduce EAAT3 FRET result measured in A430C/E57H/R61H in different ion/substrate environments. The rates between the “To” and “Ti”, or “ToNa2H” and “TiNa2H” transitions are very slow compared to the rates of the K⁺ translocation step or the Na⁺/glutamate translocation step, so for example with NaCl and 100µM glutamate outside, the transporter will favor glutamate uptake and K⁺ countertransport.

**Figure 3.13 EAAT3 model can simulate the FRET data of A430C/E57H/R61H.** A) a 15 states model of EAAT3 developed to simulate our FRET data based on previous study and models. B) Simulated voltage dependent FRET for EAAT3 A430C/E57H/R61H. The intracellular condition is set as containing 10mM NaCl, 10mM glutamate and 88mM KCl. The extracellular conditions are set as containing 100mM KCl, 100mM NaCl, 100mM Choline, 100mM NaCl and 100µM glutamate or 100mM NaCl and 100µM L-TBOA respectively.

In our FRET experiment, the measured distance represents the distance between the fluorescent head region of fluorescein and Ni²⁺ ion bound at the engineered
dihistidine site. Therefore due to the size and spatial position of the fluorophore in the protein structure, the distance measured in FRET is not the β-carbon distance in the crystal structure. The intrinsic difference between EAAT3 and Gltph structures could also result in differences in distance measured from FRET analysis compared with Gltph structures. In addition, in macroscopic FRET experiments, the measured FRET efficiency is averaged from multiple transporters potentially distributed in different conformations, while crystal structure is captured in a single state. To further investigate the time-dependent conformation dynamics of human glutamate transporter in physiological environments, in future studies the transitional metal FRET analysis developed in this study could potentially be extended into the single-molecule level, to unveil the kinetics of glutamate transporters.

3.4 Methods

Molecular Biology: Site-directed mutagenesis of the human EAAT3, in vitro synthesis of RNA, and RNA injection into *Xenopus laevis* oocytes are performed as described previously (Larsson et al., 2004).

Electrophysiology: Glutamate-induced currents are calculated by subtracting the current measured in glutamate-free Na⁺ Ringer’s solution from the two-electrode voltage-clamp current measured in Na⁺ Ringer’s solution containing 1mM glutamate as described previously (Larsson et al., 2004).

Voltage Clamp Fluorometry and FRET: VCF experiments are performed as described previously (Larsson et al., 2004). Oocytes were labeled for 30 min with 10 μM Fluorescein-5-maleimide (Molecular Probes) in Na⁺ Ringer’s solution. Fluorescence is
monitored through a FITC filter cube: exciter, HQ480/40; dichroic, Q505LP; and emitter, HQ535/50. Fluorescence intensities are low-pass filtered at 200–500 Hz and digitized at 1 kHz. The Ringer’s solution we used in this study contains 1.8mM MgCl₂, 1mM CaCl₂, 5mM HEPES with either 100mM NaCl, 100mM KCl, or 100mM choline. For all solutions in this study pH is adjusted to 7.5 with NMDG.

Fluorescence Data Analysis: Data for EAAT3 constructs are fitted with a two-site binding curve model with the following equation to account for nonspecific solution quenching of the fluorophore:

\[
\frac{F_{\text{metal}}}{F} = \left( 1 - \frac{E}{1 + \frac{K_{d1}}{[\text{metal}]} \left( 1 + \frac{1}{K_{d2}} \right)} \right)
\]

Where \( F_{\text{metal}} \) is the fluorescence of the donor in the presence of metal, \( F \) is the fluorescence of the donor without metal, and \( K_{d1} \) is the equilibrium dissociation constant for collisional quenching. \( E \) is the FRET efficiency, and \( K_{d2} \) is the equilibrium dissociation constant for Ni\(^{2+} \). \( K_{d1} \) is measured as 40mM in A53C construct and is set as constant for other fittings.

The distance (\( R_B \)) between each fluorophore and the native Ni\(^{2+}\)-binding site is measured in EAAT3 constructs without the engineered metal binding site. By fitting the F5M quenching curve with the two-site binding curve model, the distance (\( R_B \)) between each fluorophore and the native Ni\(^{2+}\)-binding site is calculated using the Förster equation:

\[
R_B = R_0 \left( \frac{1}{E - 1} \right)^{1/6}
\]

\( R_B \) is the distance between the fluorophore and the
native metal ion binding site and $R_0$ is the Förster distance, for the fluorescein/Ni$^{2+}$ pair $R_0$ is 12 Å (Taraska et al., 2009a).

For constructs containing dihistidine Ni$^{2+}$ motifs in the C-helix, the data is fit with the two-site binding curve model. Our measured FRET values ($E_{total}$) are the combination of FRET to the native Ni$^{2+}$ binding site (distance of $R_B$) as well as the engineered sites (distance of $R$). Thus, the following equation for FRET between one donor and multiple acceptors can be used (Taraska et al., 2009a):

$$E_{total} = \frac{R_0^6 \left( \frac{1}{R^6} + \frac{1}{R_B^6} \right)}{1 + R_0^6 \left( \frac{1}{R^6} + \frac{1}{R_B^6} \right)}$$

This equation can be rearranged to calculate the distance ($R$) to each engineered Ni$^{2+}$-binding motif, correcting for the native site:

$$R = 6 \sqrt{\frac{1 - E_{total}}{R_0^6 \left( \frac{1}{R^6} + \frac{1}{R_B^6} \right) - 1}}$$

Although we do not yet know whether the native FRET we observed is due to one or multiple Ni$^{2+}$ ions bound to the protein, for the purposes of this correction, FRET from multiple native Ni$^{2+}$ ions is mathematically equivalent to FRET from a single native Ni$^{2+}$ ion with distance ($R_B$).
Chapter 4

General discussion

4.1 Debates on the Na3 site

In our study, based on computational simulation and functional experiments, we propose that the Na3 site in glutamate transporter is composed of the side chain of T314 and the backbone oxygen of A353, with involvement of N401 side-chain oxygens atoms (homologous to T370, A409, and N451 in EAAT3, respectively) and in direct contact with the charged β-carboxylate group of the bound substrate. Direct substrate-ion contact had been previously suggested by electrostatic mapping (Holley and Kavanaugh, 2009), and is similar to the interaction shown in the crystal structure for the LeuT transporter (Yamashita et al., 2005), where the Na⁺ and the substrate are bound together. Direct contact of Na3 with the substrate could explain the observation that the apparent affinity for different acidic amino acids depends on the nature of the cotransported cation (Menaker et al., 2006). Recently, after the publication of our work, it was further found that mutants of residues homologous to N401 and T314 both alter cation and substrate selectivity (Teichman et al., 2012). Teichman et al. found that in EAAC1 N451S (N401 in Gltph), the K(m) of this mutant for l-aspartate is increased ~30-fold. Remarkably, the increase for d-aspartate and l-glutamate was 250- and 400-fold, respectively. Moreover, the cation specificity of EAAC1 N451S was altered because sodium, but not lithium, could support transport. A similar change in cation specificity is also observed with T370S (T314 in Glpht) mutant (Teichman et al., 2012). This new finding provides
further support to our result that T314 and N401 participate in the novel Na\(^+\) site together with the bound substrate.

About the same time as our study, other groups using computational simulations or functional experiments proposed a different Na3 site, involving ligands from D312 in TM7 and T92 in TM3 (Bastug et al., 2012; Huang and Tajkhorshid, 2010; Tao et al., 2010). However, this site is suggested to be a transiently occupied site (called Na3') according to our computational simulation, because a Na\(^+\) bound at this location is electrostatically destabilized by a Na\(^+\) bound at the Na1 site. Another study also suggested a transient Na\(^+\) site, formed by a conserved aspartate residue together with a tyrosine and located at the external end of the binding pocket, through which one or more of the Na\(^+\) have to pass on their way to other binding sites (Rosental et al., 2011). With evidence for more Na\(^+\) sites than the number of transported Na\(^+\), we propose that the three transported Na\(^+\) go through one or more intermediate binding sites before finalizing their binding positions. Our MD simulations suggest the following chronicle order of Na\(^+\) and glutamate binding: The first Na\(^+\) ion binds at the Na3' site to the ion- and glutamate-free transporter. Then, the second Na\(^+\) binds to the Na1 site and the first Na\(^+\) bound at Na3' quickly (<1 ns) moves over to the Na3 site due to electrostatic repulsion between ions at the Na1 and Na3' sites. After the binding of the first two Na\(^+\) ions at the Na1 and Na3 sites, glutamate can bind. In the final step, the third Na\(^+\) ion binds at the Na2 site after glutamate and closes the extracellular gate of the transporter.

To further determine the location and function of the different proposed Na\(^+\) binding sites, additional functional and/or computational approaches are required. For example, techniques allowing for manipulation of the backbone carbonyls will be very
informative because backbone carbonyls contribute extensively to Na\(^+\) binding sites in glutamate transporters. In additions, with Na1 and Na3 sites relatively extensively investigated by far, there are not many functional studies of Na2 site. Na2 is occupied by the last Na\(^+\) ion after glutamate has bound. A study of the function role of Na\(^+\) ion bound at this site could potentially answer the important question of how the transporter coordinates the closing of the extracellular gate and then initiate the following translocation step across the cell membrane.

4.2 Future direction: Determine the sequence of conformational changes in the EAAT3 transport cycle at the single-molecule level in living cell environment

In this study, we for the first time use the transition metal FRET method to measure distance on protein expressed in a living cell membrane. Our static FRET results show different distributions of conformational states of glutamate transporter in different ion/substrate conditions. In the macroscopic FRET experiments in this study we are looking at multiple glutamate transporters, potentially distributed in multiple conformational states at the same time. This averaged FRET data will bias towards the longest-lasting conformational state of the transporter. Furthermore, fast conformational changes could be masked by the ensemble averaging in these measurements. It is also possible that each individual transporter might switch frequently between different states (flickering), thereby blurring the transitions between them and causing an averaging of all these states. However, such blurring could be avoided by using single-molecule FRET (smFRET), where each individual transporter is measured one at a time, so that the transitions between different states can be followed over time. Based on the living-cell
transition metal FRET method we developed in this study, one could in the future develop a new FRET method by combining smFRET and Total Internal Reflection Fluorescence (TIRF) Microscopy to determine the sequence of conformational changes in human glutamate transporter EAAT3 in its physiological lipid environment to unveil the dynamics and mechanisms of glutamate transporters.

Recently, single-molecule fluorescence resonance energy transfer (smFRET) methods were developed to observe and quantify time-dependent changes in archaeal glutamate transporter homologue Gltph. However, the smFRET studies were done only in transporters purified in detergent or reconstituted proteoliposomes. In addition, these studies measured Gltph dynamics at room temperature, which is far lower than the physiological temperature of Gltph. It would be more ideal to directly study human glutamate transporter dynamics in a physiological living cell environment. Based on previous study, structural rearrangements in single-membrane proteins in living cells could be imaged by Total Internal Reflection Fluorescence (TIRF) Microscope (Fig. 4.1). Therefore we propose that we will use TIRF microscope in the future to perform smFRET experiments on glutamate transporters expressed in *Xenopus* oocytes.

![Figure 4.1 Total Internal Reflection Microscopy (TIRF) system for single molecule FRET.](image)
In smFRET experiments, we will label FRET pairs at residues which show large conformational changes in our macroscopic FRET study. We will use EAAT3 V411C/E57H/R61H to study the movement of extracellular gate HP2 and use EAAT3 A430C/E57H/R61H to study the conformation change during glutamate relocation across the cell membrane. Human glutamate transporter EAAT3 will be expressed in Xenopus oocytes and labeled with fluorescein at a low concentration to fluorescently label only 1 in 100,000 transporters, to ensure optical separation of the individual fluorophores (>1µm) for single molecule measurements. The high signal to noise ratio of TIRF will enable us to detect single fluorescein molecules that are within 100-200 nm of the cover slip. Oocytes expressing fluorescein-labeled EAAT3 will be put on a cover slip and clamped at -30mV during smFRET experiments. Single EAAT3 molecules will be visualized with a TIRF Microscope equipped with a fast low noise CCD camera (Andor iXon X3 EMCCD). The change of donor fluorescein fluorescent intensity will be imaged over time in different solutions favoring different conformations of the transporter cycle: Na⁺ Ringer, K⁺ Ringer (K⁺ rises during ischemia), and Na⁺ Ringer with different concentrations of glutamate. The FRET acceptor Ni²⁺ ions will be present at 2mM in the different bath solutions to saturate the Ni²⁺ binding site. Depending on the solution, the conformation rearrangement of EAAT3 molecules will result in changes in the donor fluorescent intensity with time and will be imaged until the bleaching of the donor fluorophore (10-100s).

Because the maximum turnover rate of a EAAT3 transporter is ≈ 25 ms per cycle, we will have to sample at a relatively high sampling frequency (1 kHz) to visualize different conformations within one single transport cycle. Relatively high laser
illumination intensity will be used to maintain signal-to-noise ratios adequate at these high sampling rates to detect FRET changes. However, we will avoid using too high laser illumination, which could lead to early photobleaching of the fluorophore that would limit the observation time and the visualization of the whole transport cycle.

We expect that in single-molecule FRET experiments, the donor fluorescence intensity of a single EAAT3 transporter molecule will undergo step-wise changes (reflecting conformational changes) upon Na\(^+\) binding, glutamate binding and transport that will be directly visualized. FRET efficiency and the distance between FRET pairs will be calculated. We expect that conformational changes that are directly associated with binding of Na\(^+\) or substrate will have Na and/or substrate dependent life times. Through the histogram of FRET efficiency we will be able to estimate the dwell time of each FRET state. We will therefore directly determine which conformational changes are directly linked to Na\(^+\) binding, substrate binding, and transport. Measurements under different conditions that favor uptake (Na\(^+\)/glutamate) or release (K\(^+\)), will show whether the release of glutamate is by a reversal in time of the sequencial conformational changes that normally lead to uptake of glutamate.

In preliminary experiment (not shown), we visualized fluorescein labeled single EAAT3 glutamate transporters expressed in oocyte membrane surface under TIRF microscope. However, due to the lateral diffusion of EAAT3 transporters in the lipid membrane, we find that in single EAAT3 transporters labeled only with FRET donor fluorescein without FRET acceptor, fluorescein intensity is highly variable from frame to frame in our TIRF measurements, as if the transporters are moving in and out of the microscope focus. This will make it difficult for us to differentiate fluorescein intensity
changes caused by the moving of the transporter protein from fluorescence intensity changes caused by FRET. In this case, we will attempt to immobilize the transporters by fusing EAAT3 transporters with the C-terminal domain of the potassium channel Kv1.4 (which contains a PDZ binding motif) and coexpressing a postsynaptic protein PSK-95 (which binds PDZ domains). This strategy was used earlier to immobilize Ci-VSP (Ciona intestinalis voltage-sensor containing phosphatase), to measure single molecule fluorescence with TIRF (Ulbrich and Isacoff, 2007).

During single-molecule fluorescence imaging of a dye-labeled EAAT3 transporter, the fluorophores might undergo transient intersystem crossing of the donor fluorophore to non-fluorescent triplet dark states, often referred to as photophysical “blinking” events, which will distort our single molecule fluorescence traces. We will perform control experiments in the absence of the FRET accepter Ni^{2+} to make sure that the “blinking” events do not significantly distort the FRET measurements. During single molecule FRET imaging, the binding and unbinding of Ni^{2+} ions to the inserted histidine motif might result in a frequent return to the zero-FRET state. To differentiate zero-FRET states caused by Ni^{2+} binding and unbinding from those caused by the structural rearrangements of the transporter, we will perform control experiment: measuring the rate of Ni^{2+} binding and unbinding using the same dihistidine motif, but with the fluorophore attached to a cysteine introduced in the same α-helix (so that the fluorophore and the dihistidine motif are immobilized relative to each other).

With the single-molecule fluorescence assay to directly study the time-dependent conformation changes during the transport cycle, we will move to the next level of understanding the conformational dynamics related with the kinetics of glutamate
transporter. This new knowledge should provide insight toward the development of novel compounds with clinical application for the treatment of neurological disorders associated with abnormal glutamate homeostasis.
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