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The dual-function glutamate transporters: structure and molecular characterisation of the substrate-binding sites

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Abstract

Glutamate transporters are essential for terminating synaptic excitation and for maintaining extracellular glutamate concentrations below neurotoxic levels. These transporters also mediate a thermodynamically uncoupled chloride flux, activated by two of the molecules they transport, sodium and glutamate. Five eukaryotic glutamate transporters have been cloned and identified. They exhibit ~50% identity and this homology is even greater at the carboxyl terminal half, which is predicted to have an unusual topology. Determination of the topology shows that the carboxyl terminal part contains several transmembrane domains separated by two reentrant loops that are in close proximity to each other. We have identified several conserved amino acid residues in the carboxyl terminal half that play crucial roles in the interaction of the transporter with its substrates: sodium, potassium and glutamate. The conformation of the transporter gating the anion conductance is different from that during substrate translocation. However, there exists a dynamic equilibrium between these conformations.

Keywords: Sodium and potassium coupling; Anion conductance; Topology; Proximity relation; Sequential binding; Dynamic equilibrium

1. Introduction

Glutamate is the predominant excitatory neurotransmitter in the central nervous system. Glutamate transporters remove the transmitter from the cleft and maintain its extracellular concentrations below neurotoxic levels [1–5]. In addition, at some synapses, glutamate transporters play an important role in limiting the duration of synaptic excitation [6–9]. Glutamate uptake is an electrogenic process [10,11] in which the transmitter is cotransported with three sodium ions and a proton [3,12] followed by countertransport of a potassium ion [13–15]. In addition to this coupled flux, glutamate transporters mediate a thermodynamically uncoupled chloride flux, activated by two of the molecules they transport: sodium and glutamate [16,17]. This indicates the existence of a tight link between gating of the anion conductance and permeation of glutamate. It has been suggested that this capacity for enhancing chloride permeability could alter neuronal excitability [17].

2. The glutamate transporter family: cloning and purification

Transporters for many neurotransmitters were cloned on the assumption that they are related to the GABA [18] and norepinephrine [19] transporters [20–22]. This approach was unsuccessful for the glutamate transporter. Three different glutamate transporters were cloned using different approaches: GLAST [23], GLT-1 [24] and EAAC-1 [25]. The former two appear to be of glial [23,24,26,27], the latter of neuronal origin [25,28] and the same is true for the later cloned EAAT-4 [17]. Indeed, these transporters are not related to the above superfamily [23–25]. On the other hand, they are very similar to each other, displaying ~50% identity and ~60% similarity. They are also related to the proton-coupled glutamate transporter from *E. coli* and other bacteria (glt-P, [29]) and the dicarboxylate transporter (dct-A, [30]) of *Rhizobium meliloti*. In these cases the identities are around 25–30%. It has been shown that the glutamate transporter family also encodes sodium-dependent transporters that do not use dicarboxylic acids as substrates, but rather neutral amino acids. These transporters are named ASCT-1 [31,32] and ASCT-2 [33,34]. The three human homologues of the rat and rabbit gluta-

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mate transporters have been cloned [35], as have two novel subtypes that are characterised by a large substrate-induced chloride current [17,36]. A smaller substrate-gated anion current, which is not thermodynamically coupled to glutamate transport, has been observed in several of the other subtypes as well [16] and also in ASCT-1 [37].

GLT-1 was reconstituted and purified [26,38] and it has 573 amino acids and a relative molecular mass of 64 kDa, in good agreement with the value of 65 kDa of the purified and deglycosylated transporter [26].

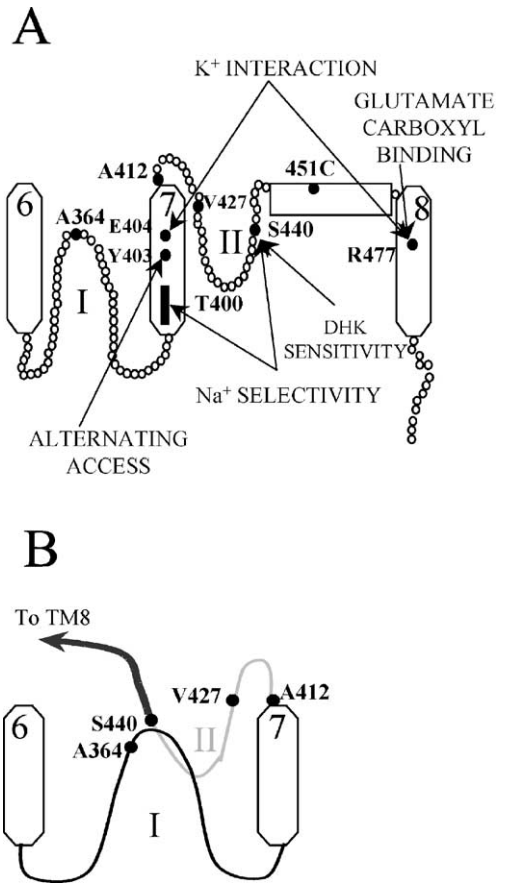

Fig. 1. Topology and proximity in the carboxyl-terminal half with the position of identified substrate interacting residues indicated. Topological model of the carboxyl-terminal half determined by accessibility to mutated cysteines (A) and a refined model determined by proximity of engineered cysteine pairs (B). The figure is adapted from Ref. [41] where the first five transmembrane domains also are shown. The structural elements in the carboxyl-terminal half include, three membrane-spanning α-helical transmembrane domains (6, 7 and 8) that are separated by two-reentrant loops (I and II) and a hydrophobic linker (horizontal box). (A) Residues that are involved in interactions with substrates and the non-transportable substrate-analogue dihydrokainate (DHK) are indicated, as well as the residues, A364, A412, V427 and S440 that are close in space (see B). Indicated is also residue 451C in the linker, which upon chemical modification leaves the uncoupled anion conductance intact but results in abolishment of the coupled uptake. (B) Two cysteine-pairs, S440C/A364C and V427C/A412C, can form a disulfide bond and must be close to each other in space [45].

3. Secondary structure and proximity relations

Hydropathy plots are relatively straightforward at the amino terminal side of the protein and the three different groups, which originally cloned GLAST-1, GLT-1 and EAAC-1, have predicted six transmembrane α-helices at very similar positions [23–25]. On the other hand, there is much more ambiguity at the carboxyl side where zero [23], two [24] or four [25] α-helices have been predicted. In the last years, attempts have been made to determine the topology of the glutamate transporters experimentally. Studies of the highly conserved carboxyl terminal half of the glutamate transporters indicate a nonconventional topology (Fig. 1A) containing two reentrant loops, two transmembrane domains (7 and 8) long enough to span the membrane as α-helices as well as an outward-facing hydrophobic linker [39–41]. Another study arrives at a somewhat different model including the assignment of transmembrane domain 7 as a reentrant loop [42]. These models disagree only on the accessibility of only one of the engineered cysteines to impermeant sulfhydryl reagents [41,43].

Accessibility studies have shown that cysteine residues introduced at positions 364 and 440 of GLT-1, located on reentrant loops I and II, respectively, react with the impermeant sulfhydryl reagent MTSET added from the extracellular side [41,44]. Substrates and nontransportable analogues partially protected against the modification of cysteines introduced at these positions. Therefore, we have suggested that positions 364 and 440 may be close in the three-dimensional structure of the protein [41]. To verify this prediction and to obtain the first information regarding the tertiary structure of the carboxyl-terminal half of the glutamate transporters, we set out to determine proximity relations between the different structural elements in this region. Two types of functional assays were used to infer proximity of engineered cysteine pairs. We identified two cysteine pairs, A412C/V427C and A364C/S440C, which behave as if they are close in space. The data provide evidence that the two oppositely oriented reentrant loops are spatially close to one another (Fig. 1B and Ref. [45]).

4. Structure–function relations of the glutamate transporters

Substrate-induced conformational changes in the GLT-1 transporter have been detected, as revealed by the altered accessibility of trypsin sensitive sites to the protease [46]. These experiments indicate that lithium can occupy at least one of the sodium ion binding sites and also that there are at least two transporter-glutamate bound states [46]. However, lithium by itself cannot support coupled transport, so one of the sodium binding sites in GLT-1 is specific; lithium cannot bind to it.

Two adjacent amino acid residues of GLT-1 located in transmembrane domain 7, tyrosine-403 and glutamate-404,

appear to be involved in potassium binding [15,47] and are close to one of the sodium binding sites (Ref. [47]; c.f. Fig. 1A). Because of the sequential nature of the transport process (Refs. [13–15]; c.f. Fig. 2), mutations in these residues cause the transporter to be locked in an obligatory exchange mode [15,47]. Moreover, tyrosine-403 behaves as if it is alternately accessible to either side of the membrane [48]. Analysis of GLT-1 mutants where serine-440, located in one of the reentrant loops, has been mutated indicates that at least part of this loop is crucial for the coupling of sodium and glutamate fluxes and that it is close to the glutamate binding site [44]. When serine-440 is changed to glycine, the GLT-1 mutant catalyses coupled transport in the presence of lithium, as if the specific sodium site now has become more promiscuous; the transporter can now accept lithium at all three sites [44]. Glycine occupies the corresponding position (G410) in the glutamate transporter EAAC-1. This transporter is able to catalyze uptake of glutamate in the sole presence of lithium [49]. Importantly, the reciprocal EAAC-1 mutant, G410S, has lost the ability of lithium to support uptake, but sodium is still able to so [49]. Another EAAC-1 mutant, T370S, has the same phenotype as G410S [49]. It appears that this conserved threonine (which corresponds to T400 in GLT-1; c.f. Fig. 1A) controls the selectivity of one of the promiscuous sodium sites; mutation to serine causes the site to become sodium-selective and lithium can no longer support coupled uptake.

We have identified an arginine residue that controls the binding of the gamma-carboxyl group of glutamate [50]. We reasoned that mutation of this arginine in a glutamate transporter that also transports a non-dicarboxylic acid

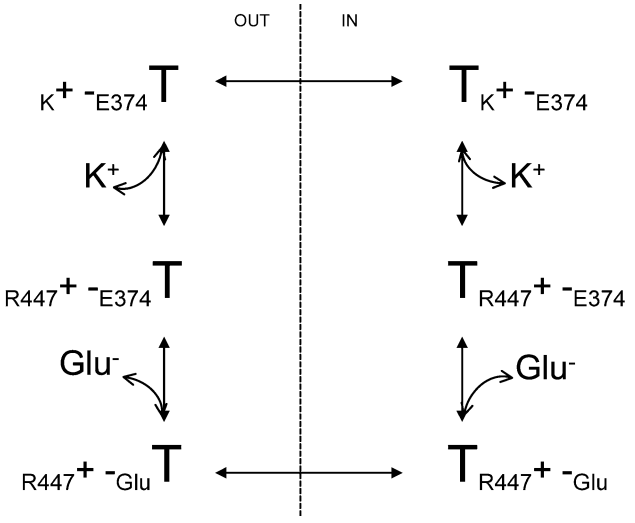

Fig. 2. Possible steps of arginine-447 interaction with glutamate and potassium. In its empty configuration Arginine-447 and Glutamate-374 interact via ion-pairing T_{R447^+-E374}. Glutamate$^-$ will take the place of residue 374 and will coordinate to Arginine-447 (T_{R447^+-Glu}). In the absence of glutamate (after glutamate debinding), potassium can take the place of residue R447 and will form an ion pair with Glutamate-374, whereupon the transporter reorients with the potassium ion. Further details are provided in the text. Reprinted from Ref. [55].

substrate might leave the transport of this latter substrate intact. It was shown that EAAT-3, the human homologue of EAAC-1, also exhibits considerable transport of cysteine [51]. We have found that mutation of the equivalent arginine-447 of EAAC-1 to neutral or negative amino acid residues completely abolishes transport of L-glutamate and D- and L-aspartate, without impairing cysteine transport. Surprisingly, this cysteine transport is electroneutral rather than electrogenic. This appears to be due to a defective interaction with potassium [50]. We propose that arginine-447, by sequentially participating in the binding of glutamate and potassium, is enabling the coupling of their fluxes [50]. The hypothetical scheme shown in Fig. 2 illustrates a possible scenario whereby this may be happening. Glutamate-374 is the EAAC-1 equivalent of glutamate-404 of GLT-1, shown to be crucial for interaction of the transporter with potassium [15]. Arginine-447 could sequentially interact with the endogenous glutamate-374 (in the unloaded transporter) or with the exogenous glutamate—the substrate to be transported (in the glutamate loaded form of the transporter). After release of glutamate on the inside, a potassium ion takes the place of the arginine cation in forming an ion pair with glutamate-374. After translocation of the potassium-loaded transporter back to the outside, potassium dissociates and arginine-447 takes its place again. In a mutant where this arginine is replaced, it is possible that glutamate-374 interacts with other residues of the transporter such that the potassium ion cannot approach it any more. This mutant can still recognise and transport cysteine. However, because of the defective interaction with potassium, the transporter would be locked in the exchange mode—only the lower half cycle would operate (Fig. 2). Obviously this is only a framework for future experimentation to unravel the coupling mechanism in glutamate transporters.

5. Relation between coupled and uncoupled fluxes

In addition to the ion-coupled glutamate translocation, glutamate transporters mediate a thermodynamically uncoupled chloride flux. It is activated by two of the molecules they transport, sodium and glutamate [16,17]. In a very recent study [49], we have reported that in EAAC-1, lithium can replace sodium in the coupled uptake but not in its capacity to gate the glutamate-dependent anion conductance. This finding suggests that the conformation gating the anion conductance may be different from that during substrate translocation. If this idea is correct, it should be possible to selectively perturb one of the two fluxes mediated by the glutamate transporters. Indeed, analysis of a mutant with a cysteine residue introduced in the hydrophobic linker region, I421C (corresponding to position 451 in GLT-1; c.f. Fig. 1A), reveals that the coupled uptake in this mutant is very sensitive to sulfhydryl reagents [52]. Strikingly, the substrate-induced anion conductance is not

affected at all by the sulfhydryl reagents [52]. Similar observations have been made on single cysteine mutants of the transporter EAAT-1/GLAST-1 at positions corresponding to positions 447 [53] and 450 [54] of GLT-1. Under conditions where the uncoupled current is dominant, sulfhydryl reagents cause a more than fourfold stimulation of this current [52]. Thus, the modification of the cysteine introduced at position 421 impacts the coupled but not the uncoupled flux. Although both fluxes are activated by substrate, they behave as independent processes that are in dynamic equilibrium [52].

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