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Minireview

The structure of glutamate transporters shows channel-like features

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Abstract Neuronal and glial glutamate transporters remove the excitatory neurotransmitter glutamate from the synaptic cleft and thus prevent neurotoxicity. The proteins belong to a large family of secondary transporters, which includes transporters from a variety of bacterial, archaeal and eukaryotic organisms. The transporters consist of eight membrane-spanning α-helices and two pore-loop structures, which are unique among secondary transporters but may resemble pore-loops found in ion channels. Another distinctive structural feature is the presence of a highly amphipathic membrane-spanning α-helix that provides a hydrophilic path through the membrane. The unusual structural features of the transporters are discussed in relation to their function.

Key words: Glutamate transporter; Neurotransmission; Channel; Pore-loop

1. Introduction

Neuronal and glial glutamate transporters in the mammalian central nervous system remove the excitatory neurotransmitter glutamate from the synaptic cleft. The process is driven by the free energy stored in the electrochemical gradients of Na^+ and K^+ ions over the membrane (Fig. 1). Glutamate uptake helps to keep the extracellular concentration of glutamate below neurotoxic levels and, at some synapses, to end the excitatory signal [1,2]. In addition, some transporters appear to have an extraordinary additional function as glutamate-gated chloride channels [3,4]. The first genes encoding mammalian glutamate transporters were cloned almost a decade ago. Since then, genome-sequencing projects have taken an enormous flight and it is now clear that the transporters belong to a large family of transport proteins with members in eukaryotes, bacteria and archaea [5]. Computational analyses of the amino acid sequences and hydropathy profiles of the glutamate transporters showed that the proteins form a unique structural class of membrane proteins [6]. In agreement, experimental studies confirmed that the proteins contain structural features, like water-filled pores and pore-loops, which are not found in 'regular' secondary transporters and which may be related to the channel-like properties associated with some of the transporters [7–11]. The recently proposed pentameric organization of the glutamate transporters also is reminiscent of channels rather than of transporters [12]. Knowledge of the folding of the polypeptide chain in the membrane is a prerequisite for understanding the complex function of the transporters. Several homologous glutamate transporters have been subjected to structural analysis and when the results from these studies are combined the first contours of the unusual structure of the glutamate transporters emerge [7,9–11]. In this minireview, the methods used and the data obtained are discussed and the unusual structural features of the glutamate transporters are discussed in relation to their function.

2. Amino acid sequence analysis

The public domain databases contain over 100 sequences of members of the family of glutamate transporters, originating from evolutionarily diverse organisms. Besides mammalian glutamate transporters, also transporters from lower eukaryotes, archaea and bacteria belong to this family [5]. Most of the proteins are related, either directly or via a common third member of the family, by amino acid sequence identities of at least 30%. It is well established that the three-dimensional structure of proteins is far better conserved than the amino acid sequences and that proteins with sequence identities as low as 30% have identical global structures [6,13]. Therefore, all transporters in the glutamate transporter family have the same global structure, which comprises the membrane topology. Multiple sequence alignment of the transporters reveals sequence similarity throughout the entire length of the proteins, but a stretch of about 150 residues in the C-terminal half is particularly well conserved [5]. This part of the proteins contains several sequence motifs, which are involved in recognition and/or translocation of glutamate and cotransported cations [9,14].

3. Hydropathy profile analysis

All membrane proteins that have been crystallized so far and that reside in the plasma or organellar membranes exclusively make use of membrane-spanning α-helices to cross the lipid bilayer. Each α-helix that spans the membrane consists of a stretch of approximately 20 residues with a high average hydrophobicity while the inter-helical loops that contact the water phase have lower average hydrophobicities. In hydropathy profiles this results in alternating regions of high and

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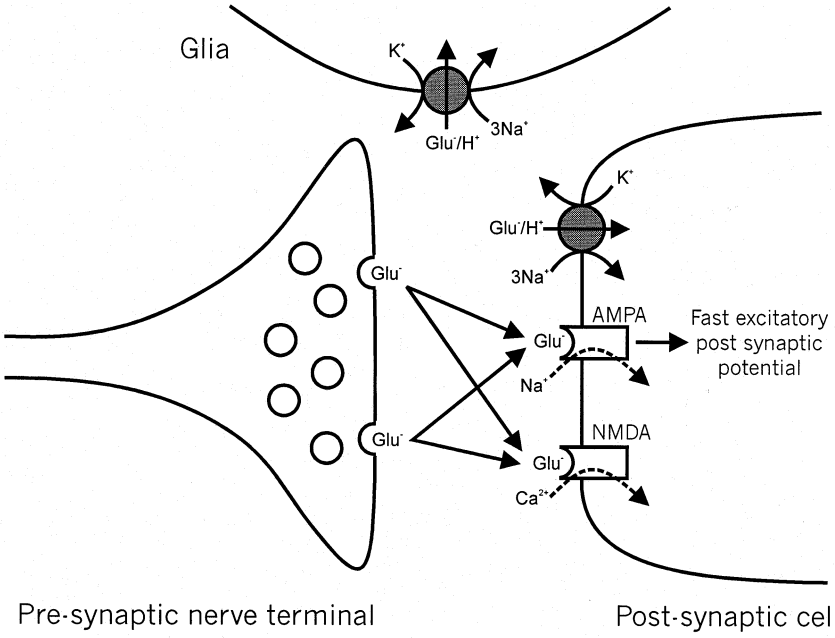

Fig. 1. Schematic representation of a glutamatergic synapse (adapted from Rothstein [25]). Glutamate is stored in membrane vesicles in the presynaptic cell [26,27] and is released by fusion of the vesicles with the plasma membrane. Subsequent stimulation of glutamate receptors (AMPA, NMDA and others) in postsynaptic cell membrane leads to a variety of responses including excitatory neurotransmission [24]. Glutamate is removed from the synaptic cleft by transporters, which are present in postsynaptic and glial plasma membranes and couple glutamate uptake with transport of protons, sodium ions and potassium ions.

low hydrophobicity (Fig. 2). The positions of membrane-spanning α-helices are recognized as peaks in the hydropathy profile when the hydrophobicity is averaged over a stretch (window) of 20 amino acids. In membrane proteins with water-filled cavities and pore-loops, such as the potassium channel KcsA [15], the mechanosensitive channel MscL [16], and glycerol and water channels that belong to the aquaporin family [17,18], the pattern of alternating regions of high and low hydrophobicity is preserved, even though some parts of the membrane-spanning helices are facing the aqueous pore. Like the membrane-spanning helices, pore-loop structures also appear as peaks in the hydropathy profile, but they are usually less hydrophobic than membrane-spanning α-helices.

The hydropathy profiles of all members of the glutamate transporter family are very well conserved, which supports the notion that the membrane topologies are the same [6]. The N-terminal half of the transporters contains six hydrophobic stretches that are typical for membrane-spanning α-helices (segments 1–6, Figs. 2 and 3). A seventh membrane-spanning segment was originally proposed at the position of segment 6a for the glutamate transporter EAAT1 [7]. However, high hydrophobicity of segment 6a is found in members of the family from vertebrate and invertebrate species, but not in the bacterial family members. The average hydrophobicity of segment 6a (Fig. 2) is not sufficient to account for an α-helix spanning the entire membrane and, in fact, the segment is part of a pore-loop structure as will be discussed below. The C-terminal part of the profiles is very hydrophobic but lacks the characteristic pattern of alternating regions of low and high hydrophobicity. It is not possible to assign membrane-spanning segments in this part, although the hydrophobic character of the region suggests membrane association. Here, experimental procedures must provide more insight.

4. Accessibility scanning

Cysteine-scanning mutagenesis [19] is currently widely used to obtain structural information of integral membrane proteins. The method makes use of mutant proteins that contain single cysteines at chosen positions. The accessibility of each single cysteine is determined with reagents that specifically react with the thiol group of cysteines. The property of the cysteine-modifying reagents that is most important for the study of membrane topologies is membrane permeability. Residues in a membrane protein that are accessible from the extracellular side of the membrane can be modified with both membrane-impermeable and -permeable reagents in whole cells. Positions that are accessible from the cytoplasmic side of the membrane can only be modified with membrane-permeable reagents in whole cells. Residues that are buried within the protein structure are not modified at all and those facing the lipid bilayer are usually not modified because the reagents are either reactive in an aqueous environment only or poorly soluble in the lipid phase.

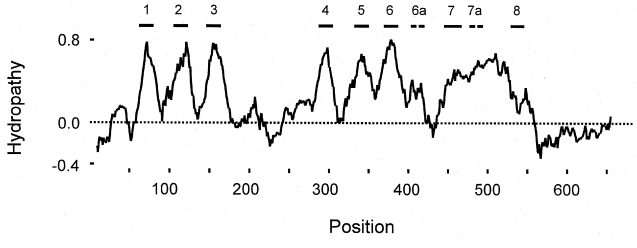

Fig. 2. Average hydropathy profile of the family of glutamate transporters based on 32 typical sequences [5]. Membrane-spanning segments and pore-loop structures are indicated by numbered horizontal bars.

The ideal membrane protein for a cysteine-scanning study consists of a tightly packed bundle of membrane-spanning α-helices, connected by flexible loops which are in contact with the aqueous surrounding and of which all residues can be labeled. Then, topology determination is straightforward. A first complication arises when modification of a cysteine is prevented by the local structure of the loop. The residue in question may not be labeled at all or different cysteine-modifying reagents may have different accessibility. As a consequence, the inability of a membrane-impermeable reagent to react with a cysteine may not be caused by the membrane barrier but by electrostatic or spatial constraints in the direct environment of the cysteine. To avoid this pitfall in topology studies it must always be shown that an intracellular residue is accessible for a membrane-impermeable reagent when the membrane barrier is removed.

Local loop structure, as opposed to global structure and membrane topology, is not necessarily conserved in evolution. Loops are the most variable regions in membrane proteins. As a result, residues at homologous positions in two members of a protein family may have different accessibility properties, even though their topological location is the same. Since the global structure is conserved in the family, it is allowed to combine positive results of labeling studies from different members of a family.

A further complication in the analysis arises when the membrane-spanning helices of the protein are not tightly packed and water-filled cavities and pore-loops are present. A residue that is positioned on the cytoplasmic side of the membrane may be accessible from the outside when a water-filled pore is present in the membrane-embedded part of the protein. In addition, residues may be accessible from both sides of the membrane, depending on the conformation of the protein, which may be dependent on the presence of substrate. Such residues are usually in regions of the protein which are involved in the catalytic activity and are detected only when the accessibility from *both* sides of the membrane is determined independently, for example in membrane vesicles with a fixed orientation [9]. Residues that are accessible from both sides of the membrane may be common in transport proteins since the substrate-binding sites have to be exposed to the extracellular and the cytoplasmic compartment alternately during the transport cycle.

5. The model

5.1. An outward-facing pore-loop

Several homologous glutamate transporters have been subjected to cysteine-scanning mutagenesis. When the results from these studies are combined the contours of an unusual structure emerge (Fig. 3). The cytoplasmic location of the N-terminus and the six membrane-spanning α-helices in the N-terminal half of the transporters as predicted by hydropathy profile analysis were experimentally confirmed in the glutamate transporter EAAT2 of rat [10]. The region between segments 6 and 7 containing the moderately hydrophobic segment 6a was predicted to be cytoplasmic. Many residues in this region were shown to be exclusively accessible from the cytoplasm, thereby confirming the location of the loop [8–10]. Segment 6a contains a very well conserved stretch of serine residues, which is extremely important for the transporter's function and which may be located close to the glutamate-binding site [9]. Cysteine-scanning mutagenesis studies in three of the family members (human EAAT1 [11], rat EAAT2 [8], and bacterial GltT [9]) have revealed that the stretch is accessible from the outside. Since the serine-rich motif is embedded in a region that is accessible from the inside, segment 6a is likely to form a pore-loop or reentrant loop which is largely intracellular but partly accessible from the outside [8,9]. Interestingly, in the glutamate transporter GltT the conserved serine-rich motif was shown to be accessible from *both* sides of the membrane. The double-sided accessibility suggests an important function in the catalytic cycle of the transporter, in which the substrate and cation-binding sides are exposed alternately to the extra- and intracellular sides of the membrane. Furthermore, large molecules (molecular weights of up to 550) can reach the motif from both sides of the membrane, indicating that the conserved serine-rich stretch faces a spacious water-filled pore [9]. Such a water-filled cavity is unprecedented in transport proteins.

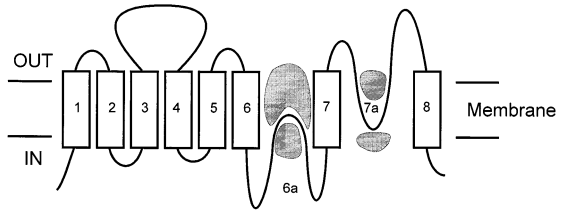

Fig. 3. Model for the membrane topology of the glutamate transporters. Rectangles indicate the positions of the membrane-spanning α-helices. Water-filled cavities are shown in gray.

The N-terminal part of membrane-spanning segment 7 is very well conserved and forms part of the binding site(s) for the cations that are cotransported with glutamate [14]. In the human glutamate transporter EAAT1 one position at the N-terminal end of segment 7 is accessible from the outside [7,11]. It is likely that the residue in EAAT1 faces the same water-filled cavity that gives access to the conserved serine-rich motif in segment 6a. This would also have important functional implications since both regions are extremely important for binding and translocation of glutamate and cotransported cations. It may be anticipated that more positions in the loop between segments 6 and 7 could be in contact with the water-filled pore in the transporters. Surprisingly, the homologous position at the N-terminal end of helix 7 in rat EAAT2 is not accessible from the outside [8]. The differences between EAAT1 and EAAT2 must reflect a different microenvironment of the residues in the two transporters.

5.2. An inward-facing pore-loop

The large hydrophobic region between segments 7 and 8 contains numerous residues that are accessible from the outside [10,11]. However, one residue in this domain in the glutamate transporter EAAT2 of rat is accessible from the cytoplasm [10]. This was explained by the presence of a second pore-loop structure, which could face a water-filled cavity. In human EAAT1 residues with cytoplasmic accessibility were not found in the same region [11] which, again, must be explained by a difference in microstructure of the loop. The non-conserved nature of the cytoplasmic accessibility of the loop suggests that cytoplasmic accessibility may not be functionally important.

5.3. A hydrophilic membrane-spanning segment

The C-terminal end of the transporters is cytoplasmic [9–11], which inevitably means that there must be an eighth membrane-spanning segment. The precise position of the membrane-embedded stretch has long been elusive because of the hydrophilic character of the region. Recently, an elaborate cysteine-scanning study has confined the membrane-spanning segment to a stretch of 23 residues [20]. When modelled as an α-helix, membrane-spanning segment 8 is highly amphipathic with a hydrophilic helical face containing numerous well-conserved polar and charged residues and a non-conserved hydrophobic face [21]. Many of the hydrophilic residues are of crucial importance for the transporter's function [20,22] and one conserved arginine was found to be directly involved in binding of the γ-carboxylate of the substrate glutamate [23]. The hydrophilic residues in the segment face an aqueous pore in the interior of the protein. The hydrophobic side of the amphipathic helix is likely to face the lipid bilayer [21].

6. Conclusions

Glutamate transporters have a unique position among secondary transport proteins with regard to their structure. The presence of two reentrant loops and a large water-filled cavity, which are crucial for the function of the transporters, is unprecedented in transport proteins. Nonetheless, glutamate transporters catalyze a reaction which is common to many secondary transporters; i.e. they use the free energy stored in the gradients of ions across the membrane to drive the uptake of the substrate glutamate. Apparently, secondary transporters must have occurred more than once in evolution. Reentrant loops have been found to play pivotal roles in the function of channel proteins. In the bacterial potassium channel KcsA reentrant loops from the four subunits of the homotetramer come together to form the selectivity filter [15]. Such arrangements are also likely to be found in related channels such as the ionotropic glutamate receptors [24]. In aquaporins, which also have a tetrameric organization, reentrant loops from different subunits do not come in close proximity [17]. Each subunit in the water channels contains two reentrant loops, an inward-facing and an outward-facing one, just like the glutamate transporters. The two reentrant loops within each subunit are in close proximity and together they form a non-continuous membrane-spanning helix, which lines the water channel. In agreement with the functional importance of the reentrant loops in the channel proteins at least one of the reentrant loops in the glutamate transporters plays a crucial role in the transport mechanism [9]. However, it is not known how the reentrant loops in the glutamate transporters are arranged. The observed pentameric organization [12] of the transporters does not exclude either one of the arrangements observed in KscA and aquaporin.

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