

Glutamate transporters: using fluorescence to determine structure and to detect conformational changes

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Abstract. Glutamate transporters remove glutamate from the synapse, thereby recycling synaptically released glutamate and maintaining a low extracellular concentration of glutamate. The molecular mechanism underlying glutamate uptake is not understood. We have used fluorescence techniques on human glutamate transporters in order to draw conclusions about the molecular mechanism of glutamate uptake. (www.actabiomedica.it)

Key words: EAAT3, FRET, VCF, sodium binding

Introduction

Removal of glutamate from the synaptic cleft by excitatory amino acid transporters (EAATs) contributes to recycling of glutamate, terminating glutamate's excitatory signal, and protecting neurons from glutamate excitotoxic injury (1). Five mammalian members of the glutamate transporter family have been cloned: EAAT1-5 (1). Recently a crystal structure of a bacterial homologue was solved (2). The structure of the bacterial homologue, Glt_{ph}, is a novel structure and surprisingly complex (Fig. 1). The transporter is a trimeric structure with a large (>50Å) extracellular vestibule that extends halfway into the membrane (2). There are 8 transmembrane domains of which some are very long and some are broken in several parts (Fig. 1A). The mechanism by which glutamate is translocated across the membrane is not clear from the crystal structure, since there is no clear aqueous pathway through the protein.

Glutamate transporters couple the uptake of one glutamate to the co-transport of 3 sodium ions and one proton, and to the counter-transport of one potassium ion (3, 4) (Fig. 1B). The exact molecular me-

chanism for this coupled transport of substrates is not well understood. An attractive model that has emerged to explain coupled transport is the alternating-access model (5). In this model, the binding of substrates to their binding sites on one side of the membrane induces a conformational change that exposes these binding sites (and their substrates) to the opposite side of the membrane (Fig. 1B). We have here used fluorescence techniques to compare the structure of the bacterial transporter to that of the human glutamate transporters and to detect conformational changes in EAATs underlying glutamate uptake.

Material and methods

Expression of EAAT3 transporters: Site-directed mutagenesis, in vitro synthesis of RNA, and RNA injection into *Xenopus laevis* oocytes were performed as described previously (6).

Voltage Clamp Fluorometry: VCF experiments were performed as described previously (6) (Fig. 2A). Oocytes were labeled for 60 min with 10 µM Alexa 546 maleimide (Molecular Probes) in sodium Ringer's

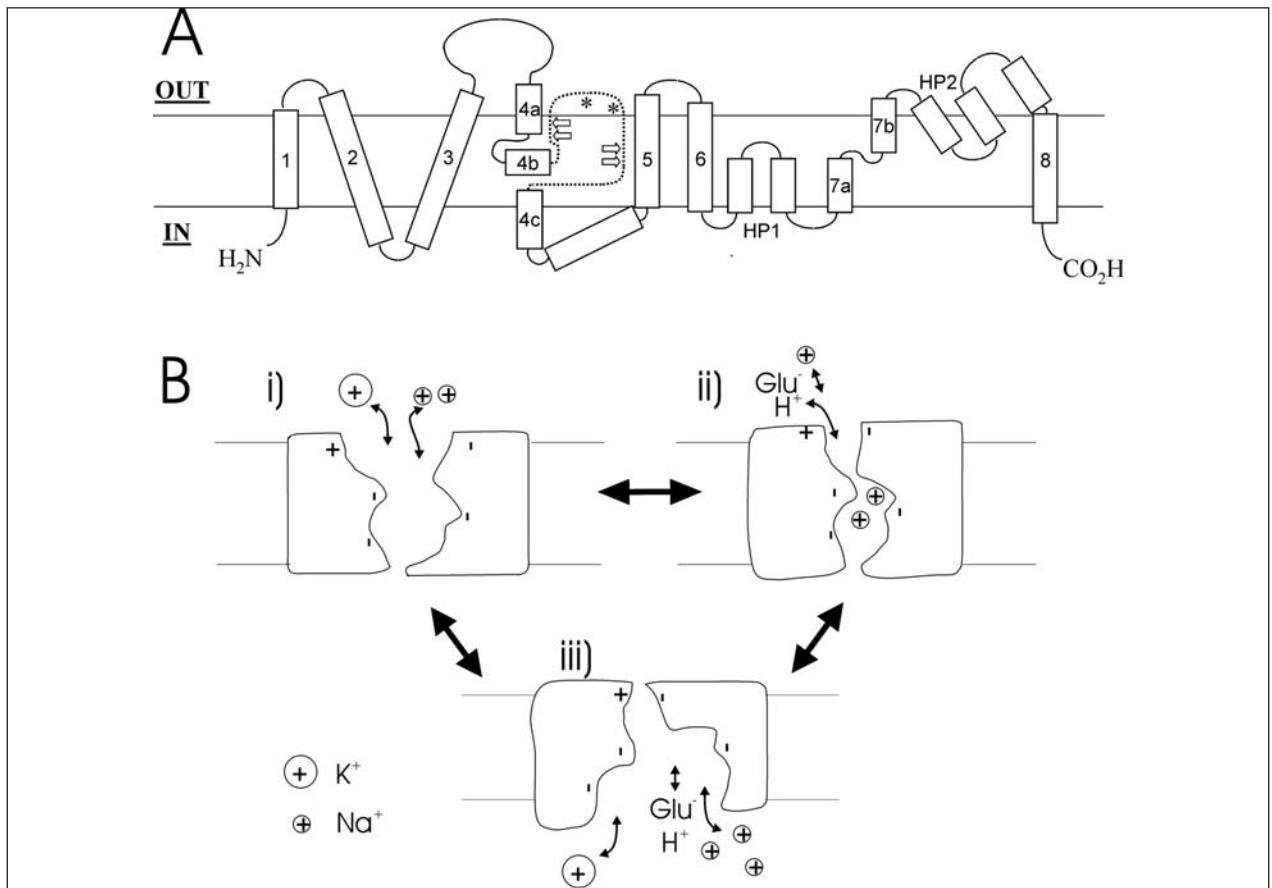


Figure 1. Topology and alternating access model for EAAT3. **A)** Topology of EAAT3 according to the bacterial crystal structure (2), showing the location of the 4b–4c loop (dotted). **B)** Alternating-access model for glutamate uptake with 3 conformational states (6): i) 2 extracellular Na⁺ bind, ii) 1 glutamate, 1 H⁺, and 1 additional Na⁺ bind, iii) intracellular release of glutamate, H⁺, and 3 Na⁺, followed by binding of 1 K⁺

solution. Fluorescence changes were recorded under Two-electrode voltage clamp using an upright microscope (Leica) with a x20 quartz objective and a photo-diode (pin-020A, UDT Sensors, Inc., CA). The objective was focused on the animal pole, and fluorescence was monitored through a rhodamine filter cube: exciter, HQ545/x30; dichroic, Q570LP; and emitter, HQ620/60m.

FRET measurements: Inter-subunit FRET was measured as described previously (7). Briefly, we first measured the labeling kinetics for each residue with donor fluorophore (Alexa-488-maleimide: Molecular Probes). Oocytes expressing the individual mutant transporters were then labeled to 10% of maximum

fluorescence with Alexa-488 in sodium Ringer's solution, which resulted in the majority of transporters having one subunit labeled with donor fluorophore. Next, the fluorescence spectrum from the donor-labeled oocyte was measured on a Zeiss LSM 510 confocal microscope, using the Zeiss META detector and exciting with a 488 nm Argon laser. The oocyte was then labeled to saturation with Tetra-methyl-Rhodamine-MTS (Toronto Research Chemicals, Toronto, Canada), and the fluorescence spectrum was measured again after the non-bound Rhodamine was washed out. To measure inter-subunit movement, spectra were measured in a sodium-free solution (98.5 mM CholineCl, 5 mM HEPES, 1.8 mM CaCl₂, and 1

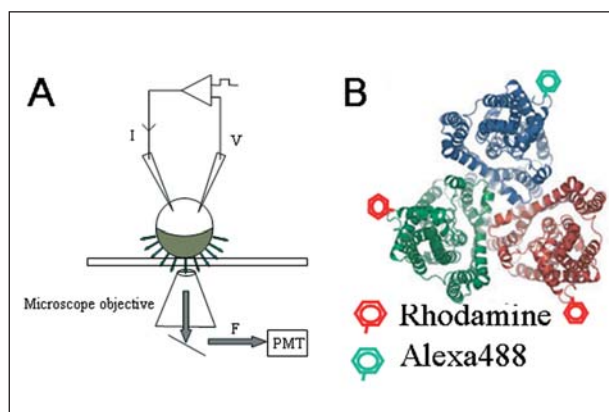


Figure 2. Fluorescence measurements from oocytes. A) Experimental set-up for VCF recording with simultaneous current (I) and fluorescence (F) measurements. B) Intersubunit distance estimates using FRET between one donor (green) and two acceptor (red) fluorophores.

mM $MgCl_2$; pH=7.5), and a sodium Ringer's solution with 1 mM glutamate, before and after the Rhodamine application. FRET efficiencies were measured as the decrease of the donor (Alexa-488) fluorescence caused by the acceptor fluorophores (Rhodamine) (Fig. 2B).

Outcomes

Using FRET as a molecular ruler, we previously showed that the human EAAT3 subtype has a similar structure to the bacterial transporter GLT_{ph} (7). EAAT3 contains a 30 amino acid extra loop between TM4b and TM4c. This loop (4b-4c loop), which is absent in GLT_{ph}, has been hypothesized to be located inside the vestibule (2). We tested the location of the 4b-4c loop, using FRET. Preliminary FRET results suggest that this loop is not in a central location, but located towards the perimeter of EAAT3 transporter. In addition, the FRET experiments suggest that there are no large conformational changes in the 4b-4c loop or any other domains in EAAT3 (Fig. 3).

Using VCF on fluorophores attached to residues in the 4b-4c loop, we show that these residues undergo conformational changes during sodium and glutamate binding. Earlier studies have shown that sodium binding is voltage dependent in EAAT3 (8). Surprisingly, the fluorescence from residues in the 4b-4c

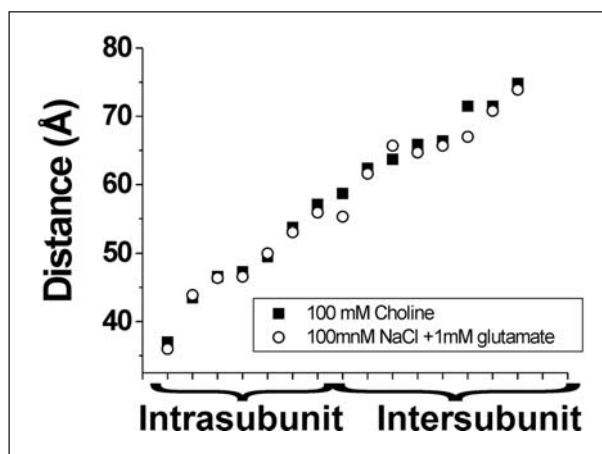


Figure 3. No large conformational changes in EAAT3. No large (>5 Å) changes in inter- and intra-subunit distances using FRET between EAAT3 residues in 100 mM choline (■) and 100mM NaCl + 1 mM glutamate (○), suggesting that glutamate uptake is accomplished without large conformational changes in EAAT3

loop reported on both voltage-dependent and voltage-independent sodium binding.

Conclusions

The structure of glutamate transporters is conserved between bacterial and mammalian transporters. No large-scale conformational changes were found in EAAT3 during transport, suggesting that glutamate uptake is accomplished by small local conformational changes that expose alternately the glutamate binding sites to intra- and extracellular solutions. There is both voltage-dependent and voltage-independent sodium binding to EAAT3.

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