Surprisingly, in contrast to the large immobilizing effect observed upon the addition of *extraliposomal* inhibitor 792949, the inclusion of *intraliposomal* inhibitor caused only a minor spectral change. This observation indicates that binding of 792949 to CTP from the internal surface of the proteoliposomes (i.e., the *matrix-facing* conformation) occurs to a much lesser extent than does binding to CTP from the external surface (i.e., the *cytosolic-facing* conformation). We conclude that external 792949 affects spin-label mobility at both monomers within the functional homodimer suggesting a tight coordination of the two monomers. Supported by NIH grant GM-054642 to R.S.K.

3574-Pos

Characterization of Substrate Binding by the Bacterial Aspartate Transporter Glt_{Ph} Through Equilibrium and Stopped-Flow Tryptophan Fluorescence Measurements

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Excitatory amino acid transporters (EAATs) remove glutamate from the synaptic cleft to ensure low resting glutamate concentrations and to terminate glutamatergic synaptic transmission. We here study substrate binding to a bacterial EAAT paralog with known structure, Glt_{Ph} from *Pyrococcus horikoshii*, using fluorescence spectroscopy. We expressed mutant transporter with an inserted tryptophan in the TM3-TM4-linker, L130W Glt_{Ph}, and studied tryptophan fluorescence of solubilized and purified L130W Glt_{Ph}. In the presence of Na^+, addition of aspartate causes changes in fluorescence intensities, as does addition of Na^+ in the presence of aspartate, allowing the construction of aspartate and Na^+ binding isotherms. Titrations of the mutant protein with aspartate at increasing temperatures resulted in increased apparent dissociation constants. Van't Hoff plots were linear over a measured range from 10 to 40°C, yielding a large negative aspartate binding enthalpy that is partly compensated by a negative binding entropy. Na^+ binding equilibria were less temperature dependent, but Na^+ binding showed to be enthalpy driven as well. Pre-equilibrium kinetics of substrate binding were monitored by measuring fluorescent changes after rapid application of substrates to L130W Glt_{Ph}. Exponential fits to the binding transients required two time constants τ_1 and τ_2, reflecting at least two underlying processes. Most of the change in fluorescence was associated with the fast process with τ_1 in the range of hundreds of ms. ${\tau_1}^{-1}$ showed linear dependence on [Na^+], suggesting that the fast process represents Na^+ binding. Aspartate uptake by Glt_{Ph} was recently shown to exhibit a Q_{10} of 3.7, indicating at least one significant conformational change during the transport cycle (Ryan et al. (2009) J Biol Chem 284, 17540-17548.). τ_1 was decreased by rising temperatures, but with lower Q_{10} than the whole transport cycle.

3575-Pos

Detection of Substrate-Dependent Conformational Changes in HP1 of the Glutamate Transporter Glt_{PH}

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Glutamate acts as the primary excitatory neurotransmitter in the mammalian central nervous system. Clearance of this neurotransmitter from the synapse is accomplished by a family of glutamate transporters known as EAATs, which move synaptic glutamate across the cell membrane into the cell against its concentration gradient. It has long been speculated that the mechanism for transport involves the movement of extracellular and intracellular gates, providing ''alternating access'' to a substrate binding site. Recently, the crystal structure of a related bacterial transporter, Glt_{Ph}, was solved, revealing two helical hairpins (HP1 and HP2) which have been proposed to contribute to these gates. A number of studies have shown that HP2, which lies on the extracellular side of the protein, can adopt multiple conformations that either provide or restrict access to the substrate binding site. However, to date there is no structural information describing conformational changes involving HP1. Here we use the technique of site-directed spin labeling (SDSL) electron paramagnetic resonance (EPR) spectroscopy to explore the local structure and dynamics of residues within HP1 (residues 264-283) in purified GltPh reconstituted into proteoliposomes. The EPR spectra suggest that the protein exists in two conformational states under our purification conditions. Upon addition of substrate, we note changes in the relative abundance of these states. We are currently working to further characterize each of these conformational states in order to better understand the structural dynamics associated with substrate transport.

3576-Pos

Mechanism of Interaction of the Glutamate Transporter EAAC1 with K^+

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Forward glutamate transport by the excitatory amino acid carrier EAAC1 is coupled to the inward movement of three Na^+ and one H^+, and the outward movement of one K^+ ion. Internal K^+ is known to bind to the transporter after glutamate and Na^+ are unloaded to the cytosol, subsequently initiating relocation of the transporter binding sites to complete the transport cycle. However, parameters of K^+ interaction with EAAC1, such as affinity and voltage dependence, are currently unknown. Here, we determined the steady-state and presteady state kinetics of the interaction of K^+ with its extracellular binding site, and the subsequent K^+ transport step, by using transport current recording from EAAC1-transfected cells. Our results show that K^+ binds to its extracellular binding site with high affinity ($K_m = 4.5$ mM). K^+ affinity is only weakly voltage dependent. However, transient transport currents were observed in response to steps of the transmembrane potential when K^+ was the only cation present. These currents were capacitive in nature and the charge movement followed a Boltzmann-like voltage dependence. Together, these results suggest that the cation binding process senses little of the transmembrane electric field, but that a subsequent K^+-induced reaction step, possibly the K^+-dependent transporter relocation, is electrogenic. The rate constant of the voltage dependent step was 70 s^{-1}. This result is consistent with previous data that suggested the K^+-induced relocation to be the rate-limiting step in the transport cycle. We propose a kinetic model, which is based on an alternating access mechanism, including a fast, voltage-independent K^+ binding step and a slow, electrogenic conformational change. Our model can be used to predict the kinetics of the K^+-dependent half-cycle of the glutamate transport process.
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3577-Pos

Sequence of Events in the Extracellular Half of the Transport Cycle in Glutamate Transporter

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The crystal structure of GltPh, a bacterial homologue of glutamate transporter (GlT), revealed the structure of the outward-facing occluded state including the substrate and two Na+ (Na1 and Na2). It has been well established, however, that substrate transport in GltPh is catalyzed by the co-transport of three Na+ ions. However, the location of the third Na+ (Na3) binding site remains unknown. Furthermore, only little is known regarding the sequence of binding events of the substrate and the co-transported ions to GltPh. In the present study, we investigate the binding sequence of substrate and Na+ ions to their extracellular binding sites using molecular dynamics simulations of various bound states of the transporter characterizing the solvent accessibility of key residues involved in ion binding and identifying the resulting conformational changes in the transporter. The results show that extracellular water cannot access Asp312 (the putative Na3 binding site) in the apo and substrate-bound states, and that this residue becomes only accessible from the extracellular side upon Na+ binding to the Na1 binding site. Based on the simulations, we propose that Na3 binds first to the Na1 binding site in the apo state, resulting in hydration of Asp312, and then moves into the Na3 binding site, the latter step likely being driven by membrane potential. The subsequent binding of a second Na+ ion (Na1) and the substrate results in a partial closure of the extracellular gate and the formation of the Na2 binding site. Finally, Na2 enters its binding site and locks the extracellular gate resulting in formation of the occluded state. We also propose a putative Na3 binding site composed of three highly conserved residues, namely, Asp312, Thr92 and Asn310.

3578-Pos

Structural Transition Between the Ion-Releasing and Ion-Binding States of a Secondary Membrane Transporter

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The crystal structure of Na^+-coupled galactose symporter (vSGLT) reports the transporter in its substrate-bound state, with a Na^+ ion modeled in a binding site corresponding to that of a homologous protein, leucine transporter (LeuT). In molecular dynamics simulations, however, we find the Na^+ ion instable, invariably and spontaneously diffusing out of the transporter through a pathway lined by D189, which appears to facilitate the diffusion of the ion toward the cytoplasm. Further analysis of the trajectories and close structural examination, in particular comparison of the Na^+ binding sites of vSGLT and LeuT, strongly indicates that the crystal structure of vSGLT actually represents an ion-releasing state of the transporter. The observed dynamics of the Na^+ ion, in contrast to the substrate, in a 200 ns equilibrium simulation, also suggests that the cytoplasmic release of the Na^+ ion precedes that of the substrate.
Through comparison of the ''open'' conformation of the Na^+ binding site in vSGLT and the ''close'' conformation in LeuT, we used constrained simulation to develop a model for the ion-binding state in vSGLT. SMD simulations were then used to pull out substrate from the substrate-binding site both in