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Sulfhydryl Modification Of Cysteine Substitutions In The Second Hairpin Loop (HP2) Alters The Ion Permeation Properties Of The Glutamate Transporter, EAAT1

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In the mammalian CNS, excitatory amino acid transporters (EAATs) limit glutamatergic signalling and regulate extracellular glutamate concentrations. EAATs have been shown to function not only as secondary active transporters, but also as anion-selective channels that regulate excitability. Under some conditions, EAATs have also been shown to mediate cation-selective conductances. The C-terminal portion of the protein contains two membrane spanning domains (TM7, TM8) and two hairpin loops (HP1, HP2), which are critical for substrate translocation, however, the anion permeation pathway has been linked to additional domains within the N-terminus of the protein (TM2). Application of bulky MTS-reagents to several cysteine substitution mutants in HP2 abolishes glutamate translocation, but preserves the anion conductance, suggesting that the anion permeation and substrate transport pathways are physically separated. Interestingly, MTS-modification of some of these mutants results in substantially greater macroscopic current amplitude. We have used two-electrode voltage clamp in Xenopus oocytes and whole-cell patch-clamp in mammalian cells to determine the basis for the change in ion permeation properties for a series of mutants that exhibit this phenotype. We examined the substrate-gated anion currents associated with one mutant, M451C EAAT1, in Xenopus oocytes and observed a profound time- and voltage-dependent deactivation of the macroscopic current at depolarizing potentials, suggesting that MTS-modification increases channel open probability. After modification, extracellular substitution with the impermeant anion gluconate, resulted in an outward current at positive potentials, suggesting that the current includes a major cation component. We are currently exploring the relationships between the substrate translocation pathway with this novel cation current and the enhanced anion current induced by modification, with the aim of better understanding the molecular determinants involved in EAAT-associated channel gating and permeation.

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Probing the Mechanism of Substrate Recognition and Translocation in the Mammalian Glutamate Transporters

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Excitatory amino acid transporters (EAATs) located on the plasma membranes of neurons and glia, play a central role in shaping neuronal signaling by removing released glutamate, and maintaining the extracellular concentration below excitotoxic levels. This transporter family is comprised of five subtypes (EAATs1-5), which share high homology and the ability to transport the acidic amino acid substrates, L-Glutamate and L-Aspartate. Despite significant biochemical data and an X-ray structure for a substrate-bound archaeal EAAT ortholog, the mechanism by which the transporters distinguish and translocate potential substrates remains undefined. One interesting observation has been that the subtype EAAT3 displays robust L-Cysteine transport relative to the other EAATs, perhaps accounting for its importance as a primary means for the neuronal import of cysteine for glutathione synthesis. To examine the structural basis for the unique substrate selectivity of EAAT3, we assessed the actions of a cysteine analog, L-Selenocysteine, which substitutes a selenium for sulfur, on the transport kinetics of EAAT3 and EAAT2. L-Selenocysteine has a pK_a=5 and thus, at physiological pH the selenol moiety is deprotonated and negatively charged like L-Glu, while the thiol of L-Cys remains protonated and uncharged. We observed that L-Selenocysteine is a potent substrate for both EAAT3 (K_m~8μM) and EAAT2, and is a more potent inhibitor of L-Glu transport in EAAT2 (IC50~39μM) than L-Cys (IC50>1mM). This result is consistent with the idea that the apparent affinity of a potential substrate should increase as its pK_a decreases, and that the arrangement of residues in the substrate binding site may significantly influence this process. These observations are further supported by the mutagenesis of key residues implicated in substrate binding/selectivity and transport, and thus provide insight into the mechanism of substrate selectivity, binding and translocation through this family of transporters.

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Quantification Of Sensitized FRET From Fluorescent GAT1 γ-aminobutyric Acid Transporters Distinguishes Between Subsurface And Plasma Membrane Resident Oligomers And Predicts Function

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γ-aminobutyric acid transporter (mGAT1) oligomerization was studied in subcellular regions using pixel-by-pixel analysis of normalized Förster resonance energy transfer (NFRET) images. Nineteen fluorescent mGAT1 protein designs were functionally characterized with non-saturated, linear uptake assays in N2a cells. Some constructs were functionally non-distinguishable from wild-type mGAT1; others oligomerized but did not traffic correctly; and some showed deficits in both assembly and trafficking. For Fluorescent mGAT1s that possess >75% wild-type function, NFRET in the peripheral region of interest (ROI) was ≥ 140% of the value in the perinuclear ROI. NFRET amplitude distributions of pixels from wild-type functioning constructs were best fit to three Gaussians. In the peripheral ROI, the highest-NFRET component comprised ~30% of all pixels, similar to the percentage of mGAT1 from the acutely recycling pool expected to be inserted into the plasma membrane in the basal state. The high-NFRET component was absent in intracellular or peripheral ROIs of cells expressing fluorescent mGAT1 constructs with ≤65% wild-type function. Thus, pixel-by-pixel analysis of NFRET quantitatively distinguishes between subsurface and membrane-inserted transporter complexes; and mGAT1 exhibits up to three quantifiable oligomerization states. Support: NIH (DA-09121; DA-10509; NS-11756), AHA (postdoctoral fellowship to FJM).

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Van't Hoff Analysis of ATP Binding to Human P-glycoprotein (ABCB1)

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ATP binding to ABC transport proteins is an established step in the mechanism by which substrates are transported. Basal ATPase activity (activity seen in the absence of transported drugs) also facilitates multidrug transport by ABC proteins (Al-Shawi *et al.* 2003, J. Biol. Chem. *278*, 52629). Therefore, we aimed to develop a non-perturbing method for detecting structural changes of P-glycoprotein associated with ATP binding during turnover. To accomplish this we analyzed heat capacity changes during ATP binding to P-glycoprotein by generating van't Hoff relationships for K_M^{ATP} values for ATP binding and K_i^{AMPPNP} values for AMPPNP binding (non-hydrolysable analog of ATP).

The rate of ATP hydrolysis by purified P-glycoprotein was measured as a function of ATP and AMPPNP concentrations at different temperatures from 23 to 35°C, in the presence or absence of saturating concentration of drug. Plots of $\mathrm{Ln}(K_a^{ATP})$ as a function of reciprocal absolute temperature in the presence of verapamil, valinomycin, colchicine and in absence of drug were fit by a non-linear van't Hoff equation. Changes in heat capacity were derived from the fits. For basal activity, the $\mathrm{Ln}(K_a^{ATP})$ increased with reciprocal temperature. The heat capacity change was positive. In the presence of verapamil the relationship declined and the heat capacity change was negative. Results obtained with the other drugs were similar. This suggests that P-glycoprotein became more hydrated on ATP binding during basal activity and adopted a more open form. In contrast, in the presence of verapamil P-glycoprotein became less hydrated and adopted a more compact form. Overall, the data support our contention that there are two structurally distinct reaction pathways for ATP hydrolysis by ABC transporters and further help clarify the coupling mechanism of ATP hydrolysis to substrate transport. Supported by NIH grant GM52502.

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Conformational motion of the Abc transporter Msba induced by Atp hydrolysis

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MsbA transduces the free energy of ATP hydrolysis to transport lipid A across the inner membrane of Gram-negative bacteria. Studies from our laboratory using site-directed spin labeling and EPR spectroscopy have defined the conformational motion during the ATPase cycle. ATP binding and hydrolysis induces the dimerization of the two nucleotide binding domains through a 30Å relative motion. The motion is propagated to the transmembrane domain leading to the reorientation of a chamber located between the two leaflets of the transporter from inward-facing to outward-facing. In this work, we investigate whether the ATP-induced conformational changes lead to repacking of the transmembrane helices as suggested by the corrected crystals structures of MsbA. In addition to a systematic analysis of mobilities and solvent accessibilities of spin labels along helices 3 and 4, we determined relative distances between helices 1 and 3 and helices 3 and 4. The data identify flexible regions in helix 3 and are consistent with a change in distance between the helices in the ATPase cycle. A detailed comparison of the data with the crystal structures will be presented.