in several conformations, providing important insight into the structure of NSS transporters. Two Na^+ sites (Na1 and Na2) were observed in LeuT crystal structures. Na1 forms part of the substrate site and Na2 is coordinated by residues from two domains thought to move relative to each other during conformational change. Previous studies using Electron Paramagnetic Resonance (EPR), Förster Resonance Energy Transfer (FRET) and scintillation proximity binding assays have shown that Na^+ is required for substrate binding to LeuT and also induces a conformational change which closes the cytoplasmic permeation pathway. This conformational change also opens the extracellular pathway, stabilizing an outward-open LeuT confornation. The molecular mechanism by which LeuT sodium sites affect substrate binding and conformational change has not previously been addressed.
We examined the role of each Na^+ site on LeuT binding and conformation. Mutation of Na1 (N27D, T254A, N286S) decreased [^{3}H]leucine binding affinity and altered the Na^+ dependence of binding. Mutation of Na2 (T354A, S355A) also decreased leucine affinity and dramatically reduced the potency of Na^+ for stimulating leucine binding. In detergent-free *E.coli* membranes from cells expressing LeuT Na^+ site mutants, Na^+ decreased reactivity of a cysteine replacing Tyr-265 in the cytoplasmic permeation pathway, confirming previous reports that Na^+ stabilizes an outward open conformation. Mutation of Na2 dramatically reduced this effect of Na^+. Mutation of Na1 also markedly altered the Na^+ protection of Cys-265, suggesting that Na1 controls not only leucine affinity but also the conformational equilibrium. These observations were complemented by single molecule FRET measurements and molecular dynamics simulations with the Na1 and Na2 mutants.

1157-Plat
Investigating the Bacterial Glutamate Transporter Homolog GltPh with Unnatural Amino Acids
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Proper function of glutamatergic synapses requires the rapid clearance of glutamate from the synapse by Na^+-dependent transporters known as excitatory amino acid transporters (EAATs). Major advances in understanding of the molecular architecture of glutamate transporters have been provided via high-resolution crystal structures of GltPh, an archaeal homolog of EAATs, in a number of conformations. Information gleaned from these structures raises a number of questions concerning the mechanism of substrate recognition, ion selectivity, and the coupling of ionic gradients to substrate uptake. Addressing these questions requires the ability to precisely modify the proteins. Chemical synthesis is an attractive tool for examining structure-function relationships, as chemical synthesis enables the incorporation of a wide variety of unnatural amino acids in addition to facilitating facile modification of the protein backbone. Here, we report the semisynthesis of GltPh in which a segment of the protein is obtained by peptide synthesis while the rest of the protein is obtained by recombinant means. We demonstrate the utility of this methodology by unnatural mutagenesis of R397 (R447 in EAAT3), a residue previously proposed to be important for substrate-binding and transport.

1158-Plat
Single Liposomes Used to Study the Activity of Individual Transporters
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Membrane transporters regulate multiple physiological processes including the pumping of ions, peptides and other metabolites such as neurotransmitters(1-4). Though transporters are extensively studied, there are currently no techniques available to investigate their function at the single molecule level.
We have developed a fluorescence-based assay to monitor thousands of single nanoscale proteoliposomes in parallel by immobilizing them on functionalized glass surfaces (5-8). We recently extended this assay to observe in real time substrate transport mediated by one or few transporters reconstituted inside single vesicles. Here we present the development of the assay and selected results from our work with Arabidopsis thaliana H^+ -ATPase 2 (AHA2), the transporter associated with antigen processing like (TAPL), vesicular monoamine transporter 2 (VMAT2, and prokaryotic ClC Cl^-/H^+ antiporter - ClC-ec1.
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Platform: DNA Replication, Recombination, and Repair

1159-Plat
Identification of the Translocation Step of a Replicative DNA Polymerase
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Replicative DNA polymerases are molecular motors that catalyze template-directed DNA replication. In each catalytic cycle, these enzymes incorporate the correct nucleotide into the primer or growing strand releasing pyrophosphate as a product. As a result of this reaction replicative polymerases translocate along their DNA substrates in steps of one nucleotide at a time (0.34 nm). Although accurate translocation is essential for genome integrity little is known about the kinetics, energetics and integration of this process in the nucleotide addition cycle during processive DNA replication. To address these subjects we have used optical tweezers to manipulate individual Phi29 DNA polymerase-DNA complexes and measure the effect of mechanical force aiding and opposing translocation on the polymerase activity at varying nucleotide (dNTPs) concentrations. Application of controlled forces on a single polymerase biases the rates of chemical reactions involving translocation and provided quantitative information about the 'real time' kinetics of elongation and the conversion of chemical energy to motion (mechano-chemistry) during protein activity. Fits to the replication velocity dependencies on force and dNTP concentration were inconsistent with a model for movement incorporating a power stroke tightly coupled to pyrophosphate release. Instead, our data is consistent with a Brownian ratchet model in which the polymerase oscillates between the pre- and post-translocation states separated by ~0.34 nm. The post-translocation state is energetically favored only by 0.7 K_BT but it is further stabilized by the binding of the correct dNTP.

1160-Plat
Single Molecule Studies of DNA Replication Processivity Clamps
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Efficient DNA replication requires processivity clamps, ring-shaped proteins that encircle the DNA and provide an anchor for DNA polymerase. In E. coli, the β clamp is a homodimer that is arranged in a head to tail fashion. In S. cerevisiae, PCNA is a homotrimer with a similar shape to β clamp but with very little sequence homology. Even though these clamps have been studied for a long time, the association affinities for the monomers and the rate constants for monomerization are not known. Both clamps are extremely stable, with estimated binding constants in the pM to nM range, so experimentally determining the dissociation constant for these clamps is difficult. We labeled the β clamp and PCNA with either one or two TAMRA dyes at each interface. The singly-labeled clamps can fluoresce, but the doubly-labeled clamps are quenched when in an oligomeric state and can fluoresce when monomers form. In order to investigate the dynamics of the dissociation of the clamps in equilibrium conditions, we used fluorescence correlation spectroscopy (FCS) and time-correlated single photon counting (TCSPC). We found that β clamp did not dissociate appreciably at 1 nM and at room temperature, but it did when the concentration was lowered to 10 pM or when the temperature was increased to 40°C. We also found that PCNA dissociates at 1 nM over hours, with a calculated binding constant of 2935 nM^2. The differences between the two clamps may indicate that they interact with clamp loaders via different mechanisms.