

By enhancing the genetic code it is possible to incorporate unnatural amino acids (UAA) with new properties into proteins without restrictions on the site to incorporate the UAA. By using the Amber suppression method in *Xenopus* oocytes we have obtained high expression levels with the heterologously expressed voltage-gated Shaker potassium channel (Kv) harboring the fluorescent unnatural amino acid Anap at various key regions. Anap is environmentally sensitive which makes it capable of probing local conformational changes in the channel. In contrast to the traditional post-translational fluorophore labeling technique with thiol-chemistry, we are now capable of probing dynamics on the cytosolic side as well as within the membrane bilayer. This opens a wide field of structural questions to be addressed since the important dynamics usually resides inside the cell or within the membrane. Anap was thus incorporated on each side of the S4 voltage sensor as well as on the S6 cytosolic gate. With voltage-clamp fluorometry we were able to determine that the four voltage sensors activate independently while the pore opening occurs cooperatively. Against previous models, pore opening required two cooperative movements. We also simultaneously labeled both ends of the voltage sensor S4 and found that dynamics of N- and C-termini differ. The successful use of fluorescent UAAs combined with voltage-clamp fluorometry has made it possible to study internal dynamics in electrogenic membrane proteins and will find widespread application in structural biology.

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Offsetting the Electric Field Sensed by Kv Channels through Residue Substitutions on Top of S1

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Kv channel subunits consist of 6 transmembrane segments (S1-S6) whereby the S1 through S4 segments assemble into a voltage sensing domain (VSD) that detects the membrane electric field. The positively charged S4 segment forms the main component of the VSD and undergoes the largest reorientations upon a membrane de- or hyperpolarization, generating a transient gating current. The S1-S3 segments surround the S4 and facilitate the latter's movement across the hydrophobic plasma-membrane. A positive (lysine) and negative (aspartate) charge substitution scan at the extracellular end of the S1 segment in the *Shaker*-type Kv1.5 channel indicated that this region is sufficiently close to the S4 segment such that it modulates the local membrane electric field. At positions E268, E272, F273 and E276 a charge substitution or charge introduction exerted a surface charge effect and shifted the voltage dependence of channel opening accordingly. Surprisingly, these residues, which modulated the electric field, did not face the S4 in a predicted 3D structure of the Kv1.5 channel in the open state (homology model based on the crystal structure of the Kv2.1/Kv1.2 chimera). This suggests that the introduced charges affect the electrical field around the S4 segment in the closed state only. In conclusion, residues at the top of the S1 segment can state-dependently offset (polarize) the electric field sensed by the S4 segment, supporting that both segments are in close proximity. (This research was supported by fellowship CONACyT #203936 to EMM & grant FWO-G.0449.11N to DJS.)

2718-Pos Board B410

Allosteric Coupling of the Inner Activation Gate to the Outer Pore of a Potassium Channel

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In potassium channels, functional coupling of the inner and outer pore gates may result from energetic interactions between residues and conformational rearrangements that occur along a structural path between them. Here, we show that conservative mutations of a residue near the inner activation gate of the *Shaker* potassium channel (I470) modify the rate of C-type inactivation at the outer pore, pointing to this residue as part of a pathway that couples inner gate opening to changes in outer pore structure and reduction of ion flow. Because they remain equally sensitive to rises in extracellular potassium, altered inactivation rates of the mutant channels are not secondary to modified binding of potassium to the outer pore. Conservative mutations of I470 also influence the interaction of the *Shaker* N-terminus with the inner gate, keeping it open and separately promoting re-orientation of the outer pore.

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Enhancement of C-Type Inactivation by External Ca^{2+} and La^{3+}

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The ShB ΔN mutant T449A undergoes C-type inactivation after depolarization within ~100 ms in external solutions containing 2-4 mM Ca^{2+} and 0 K^+ . Raising external Ca^{2+} to 40 mM, reduces peak I_K for a single pulse to ~20%, and virtually eliminates I_K with steady pulsing at 0.1 Hz. The effect

of Ca^{2+} is reversible. Raising external K^+ to 5-20 mM (in 40 mM Ca^{2+}) largely restores I_K . How is Ca^{2+} suppression and K^+ restoration of I_K explained? Ca^{2+} blocks inward I_K through an open channel strongly at negative voltages. We propose that Ca^{2+} competes with K^+ at Site₀ (just outside the selectivity filter) where K^+ is strongly favored, but Ca^{2+} occupancy is enhanced at negative voltages. Ca^{2+} occupancy of Site₀ strongly reduces K^+ access to and occupancy of Site₁ (outermost site of the filter), allowing repulsion among the carbonyls of Site₁ to dilate this site, causing C-type inactivation. La^{3+} has almost the same crystal radius as Ca^{2+} , and is much more potent in suppressing I_K : with 10 μM La^{3+} externally, I_K of T449A is largely eliminated (reversibly). We postulate that trivalent La^{3+} has high affinity for Site₀. Neither Ca^{2+} nor La^{3+} has strong effects on T449V, which does not exhibit C-type inactivation.

2720-Pos Board B412

Shaker Kv Channel's Sugar Remotion in Real-Time

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Voltage-gated K^+ channels (Kv) are tetramers, each subunit containing six transmembrane segments (S1-S6). These segments make two functionally and structurally independent domains: an ion conduction pore (S5,S6), and voltage-sensor domains (S1-S4). Like many transmembrane proteins, Kv channels undergo extensive posttranslational modifications, with a significant percentage of the mature protein mass being composed of glycan moieties. Shaker Kv channels are glycosylated at two asparagine residues (N259 and N263) located in the first extracellular loop. Mutating asparagine residues to glutamine abolishes glycosylation but does not prevent cell surface expression of functional channels. Using the same population of Shaker Kv channels, we studied the function and toxin binding before and after deglycosylation by PNGase F. Binding properties of Hanatoxin, GxTx and Agitoxin remained unaltered by removing glycosylation moieties, suggesting that sugars attached to the channels are not intimately interacting with regions of the voltage sensor domains and the pore where these toxins bind. We also studied ionic and gating currents, before and after deglycosylation with PNGase F. Opening kinetics were substantially slowed down by deglycosylation, as reported previously in many members of the Kv family. Total charge distribution mediated by the voltage sensors of the non-conductive W434F mutant Shaker channels shifted only ~3-4 mV towards positive potentials after deglycosylation. Overall our data provides direct proof to understand how glycosylation contributes to Kv channel function.

2721-Pos Board B413

Locking the Open State of a Voltage-Dependent Concatemer Potassium Channel with Metal Bridges

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Voltage-gated K^+ (K_v) channels are homotetramers. Each subunit is formed by six transmembrane segments, S1 to S6. The permeation pathway is formed by S5, the P-region and S6 from each subunit, that assemble around a central canal through which K^+ permeate. In response to voltage changes, the voltage sensor domain (S1-S4) undergoes conformational changes that are coupled to the opening and closing of an intracellular gate located at the inner end of S6. It is known, that a cysteine at position 476 in S6 (near the gate) traps the mutant channel in the open state when Cd^{2+} is added to the intracellular solution. This behavior is caused by a metal bridge between the cysteine at 476 of one subunit and a native histidine at 486 in an adjacent subunit. We constructed a Shaker K_v concatemer that has all 4 subunits linked at the DNA level. When all four V476 are mutated to cysteine, intracellular Cd^{2+} , locked the channels open as in wild-type channels. We are presently developing concatemer channels with one, two and three metal bridges to assess one by one their contribution to the opening of the channels. Because the metal bridges can be disrupted by a H486Y mutation, this approach should allow us to discern the assembly of a functional concatemer channel in the membrane.

2722-Pos Board B414

Mutations in the Cavity Affect the Rate of Slow Inactivation in Shaker K^+ Channels

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It has been reported earlier that the activation and inactivation gates of Shaker channels are coupled, and this coupling might be mediated by a rotational motion of the S6 helix. We hypothesize that either the electrostatics or the volume/hydrophobicity of the side chains pointing into the cavity can affect this coupling. Accordingly, we have introduced side-chains at position 470 in a

T449A Shaker-IR background with different hydrophobicities according to the Wimley-White hydrophobicity scale (Ala, Cys, or Trp) and charges (Glu, Asp, Arg, Lys) and measured the inactivation kinetics. Mutant channels were transiently expressed in tsA_201 cells. All ionic current experiments were performed with excised inside-out patches. The introduction of an ionizable cysteine and the small but neutral alanine dramatically slowed entry into the inactivated state (the inactivation time constants for Shaker mutant T449A/I470C, T449A/I470A and T449A were 1.4 s, 250 ms and 50 ms, respectively). We could not detect any current on mutant T449A/I470X (X = Asp, Glu, Trp) but the transfection with a mixture of mutant and wild-type expression plasmids expressed currents. These heterotetramers had slower inactivation kinetics with respect to the T449A channels. Almost all of the channels had a midpoint voltage for activation in the range -50 mV to -20 mV. The only exception was the I470C mutant which had a large shift in voltage dependence (midpoint ~5 mV). Mutant channels containing Arg or Lys (I470R, I470K) did not give functional expression either as homotetramers or as heterotetramers. Our results show that not the size but the physicochemical properties of the side chains (hydrophobicity and charge) at position 470 determine the inactivation kinetics of voltage-gated K⁺ channels, which may reflect interaction of the side chains with permeant ions.

2723-Pos Board B415

Voltage Sensor Domain Mutations Involved in the Kv1.2 Channel Activation via MD Simulations

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Voltage-gated cation channels (VGC) are membrane-bound proteins responsible for the generation and propagation of the action potential that regulates a number of critical biological events such as skeletal muscle contraction, neuron activity and hormone secretion. These channels are equipped with voltage sensor domains (VS) that act as electrical devices highly sensitive to transmembrane (TM) voltage variations () that modulate the gating state of VGC. Despite major structural conservation within VS from the VGC family, there are marked sequence variations among them related to their diverse kinetic rates for *up-down* turnover. For instance, the VS kinetics is markedly distinct between Kv and Nav channels, a feature that complies with their respective role in the fast and slow phases of the action potential. Currently, original results concerning single point mutations have identified key amino acids directly affecting the kinetic rates of Shaker-like channels activation, such as I237A (Lacroix J.J. *et al.*, 2012), F290A (Schwaiger *et al.*, 2013), and more recently I287T/V363T (Lacroix J.J. *et al.*, 2013).

Here, by benefiting from the well understood activated and resting states of Kv1.2 we investigate the molecular nature of such mutations affecting the *up-down* VS turnover. Specifically, we use all atom MD simulations to investigate the structural effects of the referred mutations to the sensing domain. Anticipating our results, such amino acid changes provoke minor structural rearrangements in the *up* and *down* states of the VS, thus indicating that these mutations could actually be affecting the intermediate states within the activation pathway of the channel.

2724-Pos Board B416

Atomistic Modeling of Ion Conduction through Voltage-Sensing Domains

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Voltage-sensing domains (VSDs) are modular membrane protein units that sense changes in the membrane electrostatic potential, and through conformational changes, regulate a specific function. The VSDs of voltage-dependent K⁺, Na⁺ and Ca²⁺ channels do not conduct ions under physiological conditions, but they can become ion-permeable under pathological conditions through mutations in the voltage sensor, particularly of S4 basic side chains [1,2]. Relatively little is known about the underlying mechanisms of conduction through VSDs. The most detailed studies have been performed on Shaker K⁺ channel variants that include the mutation of the outermost Arg residue in S4 to a smaller, uncharged side chain [1,2]. Ion conduction through the Shaker VSD is manifested in electrophysiology experiments as a separate voltage-dependent inward current that appears when the VSDs are in their resting state conformation [1]. Only monovalent cations permeate the Shaker VSD through a narrow and twisted pathway after reaching a vestibular region on the extracellular side of the VSD [1,2]. This permeation pathway is, at least in part, the same as that one followed by the S4 basic side chains during voltage-dependent activation. We sampled VSD ion conduction events on the microsecond timescale

under a membrane potential using experimentally validated models of the Shaker VSD [3] in a resting state in order to elucidate the molecular mechanisms of ion conduction, gating, and selectivity. This work was supported by NIH Grants F30CA171717 to M.L.W. R01GM098973 to FT, P01GM86685 to DJT SHW, and P41GM103712-S1 to the National Resource for Biomedical Supercomputing.

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2725-Pos Board B417

Cys Mutation + MTS Caution is Needed in Interpretation of Arg Reaction in the S4 Transmembrane Segment of a Voltage Sensing Domain (VSD) of a Voltage Gated Channel: Results of Quantum Calculations

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In all previous work on voltage gated channels, mutation of the arginines on the S4 segment of the VSD, then reaction with an MTS reagent, followed by channel shutdown, has been taken to mean that the arginine was exposed on the surface from which the reagent was applied. This may require more care in interpretation. Cysteine is smaller than the arginine by about the size of the reactive sulfonate on the MTS; The mutation leaves a large cavity where the arginine side chain had been, so the MTS can reach the cysteine, possibly via the omega pore. The backbone atoms need not move. The distance between S4 and S2 or S3 remains largely unchanged. Salt bridges, (e.g., R297-E183) however, are disturbed; when cysteine is in the reactive (negative) form, it constitutes a charge reversal mutation, as the arginine was (presumably) positive. Quantum calculations on configurations of this region for R300C of the VSD of Kv1.2 show that the cys anion can fold away from the cavity where it could react, in a manner dependent on the water and protons present. See the preprint posted at <http://arxiv.org/abs/1309.1373>. Optimizations have been done at BLYP/6-31G** level. Acknowledgement: Computations were done at the Brookhaven National Laboratory CFN cluster, and the CUNY hpc facility.

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Quantum Calculations Show How the Water at the Gate of the Voltage Gated Kv1.2 Channel Plays a Major Role in Determining Conduction through the Gate

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We have carried out quantum calculations to optimize the structure of the gate region of a Kv1.2 (with hydrogens added as in the pdb 3Lut structure) channel as well as the structure below the gate as far as the T1 segment, starting from the X-ray structure. A key result: A PVPV->PVVV in silico mutant disrupts the water structure. The normal gate opens just enough to allow a hydrated K⁺ to enter the cavity that separates the gate from the selectivity filter. The mutation also alters the electrostatic potential. In addition, we have calculated the result of protonation of the H418 below the gate, which shows approximately a 3 Å rotation toward the pore on protonation, enough to alter the potential at the gate; deletion of H418 is reported to produce a non-functional channel(1). With Cl⁻ ion at the gate, but not K⁺, a water structure forms in the optimized channel structure, and appears to be fairly stable; this may already select against anions. Calculations in cases that involve K⁺ include bond order and charges on all atoms, enabling calculation of electrical potentials. Optimizations were done initially at HF/6-31G* level; most have been confirmed at BLYP/6-31G** level, with little change in any case tested. Acknowledgement: Computations were done at the Brookhaven National Lab CFN cluster and the CUNY hpc facility.

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Turning a Small into Large Conductance K-Channel - How Far Can We Go?

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Potassium channels are membrane proteins that allow the passage of K⁺ ions across the hydrophobic core of the membrane. They display an extremely conserved signature sequence that elicits high ion transport rates and exquisite discrimination between ions with similar radii. Despite of this conservation,