and R_{eq}^-, the photoconverted anionic chromophore R_{pc}^- exhibits a reduced fluorescence lifetime of 2.2 ns. The fluorescence lifetime which is measured in fluorescence lifetime imaging microscopy with spatial resolution depends however on the excitation conditions and history. The underlying photochemistry is described by the kinetic scheme of consecutive reactions, $R_{eq}^- -> R_{pc}^- -> P_{dark}$, in which the anionic chromophore species and the dark protein P_{dark} are coupled by photoconversion and photobleaching. Time correlated single photon counting in a confocal geometry of diffusing species is used to compute the quantum yields for photoconversion and photobleaching for the anionic chromophore species.

114-Plat Novel Two-Photon Absorption Bands in Fluorescent Proteins

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Despite a rather extensive use in high-resolution imaging of living cells and tissues [1], two-photon excitation properties of Fluorescent Proteins still need to be thoroughly investigated and characterized over a wide range of excitation wavelengths and external conditions. An optimal two-photon excitation wavelength can be often obtained by doubling the maximum one-photon excitation, and normally the two-photon excitation profile of FPs at about halved wavelength is found to mimic the one-photon excitation [2]. However, other spectral regions that are inactive at one-photon excitation can become active when accessed by two-photon excitation. DsRed, in particular, displays such "anomalous" behavior: a strong two-photon band appears at wavelengths shorter than 780nm. At the corresponding one-photon region (370nm) the one-photon absorption spectrum is almost completely flat [2,3]. By means of Density Functional Theory techniques, we explain here the presence of this band as stemming from transition to a higher excited state, through coupling with the HOMO-LUMO one-photon excitation [4]. We shall argue that two-photon excitation bands in the 500–700nm region are ubiquitous among FPs, and might provide interesting spectral windows for multiphoton fluorescence imaging.

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115-Plat Singlet Oxygen Production By Proteins From The GFP Family

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Proteins from the green fluorescent protein (GFP) family are increasingly being used as genetically-encoded reporters for intracellular dynamics, protein expression and protein-protein interaction studies based on fluorescence microscopy. Extended observation of GFPs is unfortunately limited by photobleaching/photoconversion of the chromophore or light-induced damage of the surrounding biological medium. Photoproduction of reactive oxygen species are thought to play a key role in this limitation. The protein photophysics can be fine-tuned by modification of the chromophore's environment and recently a GFP mutant has been engineered for genetically-targeted chromophore-assisted light inactivation. The prospect of using genetically-encoded photosensitizers for mechanistic and eventually therapeutic purposes has lead us to study their ability to photosensitize the production of singlet oxygen. The results of these studies reveal the role of the beta can in the photosensitization process.

Platform K: Voltage-gated K Channels, Voltage-Dependence & Gating

116-Plat Engineering Atomic Constraints Between the Voltage Sensor and the Pore Domain of a Voltage-Gated K^+ Channel of Known Structure

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Voltage-gated K^+ channels (Kv) are tetrameric subunit complexes surrounding an aqueous pore, each subunit comprising six transmembrane segments (S1–S6) with S1–S4 constituting the voltage-sensor domain (VSD) and S5–S6 the pore-forming region. Membrane depolarization promotes structural re-orientation of each of the four voltage-sensor domains allowing pore opening. Although the crystal structure of Kv1.2 provided the first atomic resolution view of a eukaryotic Kv channel, several components of the VSD remain poorly resolved. In particular, the position and orientation of the charged arginine side chains in S4 during voltage-dependent channel activation remains controversial. Here, utilizing electrophysiological analysis of intersubunit metallic bridge formation as employed previously in *Shaker*, in combination with all-atom molecular dynamic simulations of the Kv1.2 channel in an explicit membrane, we demonstrate that S4 of Kv1.2 interacts directly with the pore domain in the open-activated conformation, and we provide a structural model for this conformation. The results from electrophysiology indicate that residues R294 and A351 are closer than the 13.7 Å predicted in the Kv1.2 crystal structure, consistent with the molecular dynamics simulations.

117-Plat Disintegration Of The Voltage-sensor Paddle During Potassium-channel Gating

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Changes in membrane voltage open and close voltage-gated ion channels by moving a voltage sensor in the channel protein. The positively charged fourth transmembrane segment, S4, plays an essential role in this mechanism. A recent model suggests that S4 is fixed to the extracellular part of the third transmembrane segment, S3b. Together they form the voltage sensor paddle, which moves through the lipid bilayer to open the channel. Other gating models suggest that S4 moves alone. We investigated this by searching for pairs of mutated cysteins in S4 and S3b to make disulfide bonds. The Shaker K channel was expressed in *Xenopus* oocytes and investigated with a two-electrode voltage-clamp technique. We found a residue in S3b that makes a disulfide bond with a residue in S4. The disulfide bond can be formed and broken during the experiment. Furthermore, the bond formation is state dependent; the bond can be formed in the open state but not in the closed state. These data are not compatible with a rigid voltage sensor-paddle where S3b and S4 are fixed to each other.

118-Plat Gating-Charge Measurements of the Kv1.2 Potassium Channel

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Voltage-gated ion channels are membrane proteins that open and close in response to voltage changes across the cell membrane. Voltage-gated potassium (Kv) channels are composed of two structurally and functionally separate domains, voltage-sensing domains (VSD) and the ion conduction pore. The voltage-sensing domain is made of four transmembrane segments that are highly charged, and move in response to changes in the electric potential, and thus result in opening of the channel. The x-ray crystallographic structure of the Kv1.2 channel has provided the first view of the molecular structure of a Kv channel in its native open conformation. However, no structure is currently available for the closed state and the mechanism of voltage-gating is poorly understood. Complete models of the Kv1.2 channel in the open and closed states were developed using homology, de novo, and domain assembly methods of the structure prediction program ROSETTA, which was recently adapted to work with membrane proteins. Molecular dynamics (MD) simulations were carried out to refine the open and closed state models of Kv1.2 in an explicit water-membrane environment. The MD simulations indicate that these complete atomic models of the channel are very stable. Each model was simulated for 100ns in the presence of a positive or negative voltage using the program NAMD. The studied system consisting of the protein embedded in a patch of DPPC bilayer and 0.4M KCl solution included 350K atoms. The magnitude of the gating-charge that is transferred across the membrane upon opening of the channel was calculated from MD simulations of the VSDs (open and closed states) in the explicit water-membrane environment by measuring the difference in the displacement currents of the open and closed states when an electric potential is applied; the results show excellent agreement with the observed values.

119-Plat Loose Coupling Between The Voltage Sensor And The Activation Gate In HCN Channels Suggests A Molecular Mechanism For Voltage Gating

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Voltage-gated potassium (Kv) channels have a strong coupling ($>$109) between the voltage sensor (S4) and the activation gate: when S4s are activated the gate is open to $>$80%, but when S4s are deactivated the gate is open $<$10–9 of the time. Using noise analysis, we here show that the coupling between S4 and the gate is $<$200 in HCN channels. Locking the gate open or decreasing the coupling between S4 and the gate in HCN channels had only minor effects on the energetics of S4 movement, consistent with a weak coupling between S4 and the gate. In contrast, locking the gate open in a Kv channel drastically altered the energetics of S4 movement. We further identified a pair of interacting residues in spHCN channels that together with the loose coupling in HCN channels suggests a possible mechanism for the inverted voltage gating in HCN channels compared to in Kv channels.

120-Plat Voltage-Dependent Gating In A Voltage Sensor-Less Ion Channel

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Lacking a canonical voltage-sensing domain, the open probability (Po) of KATP and other Kir channels is regulated by multiple ligands, including cytoplasmic ATP and anionic phospholipids such as PIP2, but this gating is insensitive to changes in membrane voltage. We have identified a single point mutation (L157E) within the M2 transmembrane helix of Kir6.2 that confers significant voltage-dependence to Po. Similar to voltage-dependent Kv channels, the Kir6.2[L157E] mutant exhibits a time-dependent activation upon membrane depolarization, resulting in an outwardly-rectifying current-voltage relationship. This voltage-dependence integrates into the intrinsic ligand-dependent gating mechanisms of Kir6.2, as features of voltage-dependent gating can be modified in conditions that affect channel Po. Increasing the membrane PIP2 content saturates Po and eliminates voltage-dependence, whereas the effects of voltage are more dramatic in conditions that reduce channel Po (eg. application of ATP or poly-lysine). Both the extent and time-dependence of voltage-gating are highly sensitive to internal [K+] (Ki); with increased Ki increasing the time-dependent component of activation and decreasing Po, suggesting that K+ occupancy of the inner cavity can affect stability of the ligand-operated gate. These results describe a novel engineered gating mechanism in a ligand-dependent K+ channel, allowing for rapid and reversible changes in channel activity without altering ligand concentrations. In addition,

the findings likely describe a previously unrecognized voltage-sensing mechanism, and alter our view of the minimal structural requirements for voltage-dependent gating of an ion channel.

121-Plat Interaction Of The Voltage-Sensing Domain Of Kvap With Lipid Bilayers

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A large family of membrane proteins contain voltage-sensing domains (VSD) that allow them to respond to changes in membrane voltage. Very little is known about the topology of the VSD in the lipid bilayer, as well as the effects of VSD on the properties of the bilayer. We have begun to examine the interaction of the VSD from the KvAP potassium with lipid membranes using a range of biophysical approaches. The KvAP VSD was expressed in *E. coli*, purified in octyl glucoside (OG) and reconstituted into the proteoliposomes (POPC:POPG (1:1)). Circular Dichroism spectroscopy indicates that the VSD has high helical content, both in OG micelles and in proteoliposomes, consistent with the structure solved by X-ray crystallography (Jiang, et al. Nature 2003 *423*:33–48). The KvAP VSD contains a single Trp in the S2 helix (W70), which exhibits maximum fluorescence emission at 335 nm when the VSD is in 3 % OG or reconstituted in the lipid matrix, suggesting that W70 resides in a hydrophobic environment. Quenching of W70 fluorescence by acrylamide or potassium iodide is dramatically reduced when the VSD is in OG or the lipid matrix compared to Trp in solution, suggesting that W70 is buried. Quenching experiments with brominated lipids indicate that W70 is located close to the center of the bilayer when the VSD is reconstituted into the lipid matrix. Lipid multilayers containing the VSD were formed by deposition of proteoliposomes for neutron diffraction studies. Four orders of diffraction were observed for the proteoliposome samples at 86% relative humidity. We are currently exploring the influence of the KvAP VSD on the distribution of water, headgroup and hydrocarbon regions of the lipid bilayer.

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122-Plat Counting Pores in a Voltage-Gated Proton Channel

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The Hv1 voltage-gated proton channel belongs to the large family of proteins containing voltage-sensing domains (VSDs). In these proteins the VSDs control an effector domain, which can be an enzyme, as in Ci-VSP, or a pore domain, as in Kv, Nav and Cav channels. Unlike the other members of the family, Hv1 does not contain an effector domain. So, the proton pore must be located somewhere in the VSD. We have recently described a permeation pathway in the VSD of a mutant Shaker potassium channel, the omega pore, and asked whether there are similarities between the omega pore and the proton pore of Hv1. The finding that Hv1 channels are made of multiple VSD subunits (see abstract from Ulbrich et al.) raises the possibility for the proton pore to be located at the interface between subunits. Alternatively, each VSD subunit could contain its own permeation pathway, and therefore there could be as many pores as there are subunits. To distinguish between these two possibilities we use two complementary approaches. In the first approach, we compare the effects of cysteine-modifying reagents on the proton current carried by Hv1 channels made of:

(i) only wild-type subunits,
(ii) only mutated subunits bearing cysteine substitutions at positions laying in the permeation pathway,
(iii) mixed wild-type and mutated subunits.

In the second approach, we look for potentially functional chimeras between the multimeric Hv1 and the monomeric Ci-VSP.

123-Plat Ci-VSP Charge Movement Shows A Voltage-sensor-inactivation-like Behavior

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The N-terminus of Voltage Sensitive Phosphatase from *Ciona intestinalis* (Ci-VSP; Murata, *et al.*, 2005) contains four putative trans-membrane segments with high homology to the voltage sensing domain of Shaker K channel and when expressed in *Xenopus laevis* oocytes exhibits a voltage dependent charge movement, or "sensing" current. A single Boltzmann distribution was fitted to curves of charge *vs* potential (QV curve). The fitted midpoint ($V_{1/2}$) of the distributions was +50 mV when the holding potential (HP) was −60 mV. When the HP was +80 mV, the QV shifted toward negative potentials, with a $V_{1/2}$ around −5 mV. This feature resembles the QV curve shift observed in several voltage-gated channels that correlates with the slow inactivation of their conductance (*i.e.* Bezanilla *et al.*, 1982; Olcese *et al.*, 1997). The time course of the QV shift was measured by recording "sensing" currents after a variable duration pre-pulse to +80 mV giving a time constant around 565 ms. Fluorescence recording of TMRM labeling at position 214 (Kohout *et al.*, 2007) showed quenching with two time constants where the fast time constant was similar to the "sensing" current. The slow component of the fluorescence had a time constant of 530 ms when holding at −60 mV and pulsing to +80 mV matching the time constant for the QV curve shift observed at positive HP. These observations indicate that the observed QV shift is an intrinsic property of the voltage sensing domain. Upon depolarization, the voltage sensor of Ci-VSP moves into an active state and subsequently undergoes a slow transition into another state, stabilizing its charges in a different conformation. Because this is reminiscent of the inactivated state of voltage-gated channels, this phenomenon may reflect a general property of voltage sensing domains.

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