

**1662-Pos****Alteration of Extracellular pH Dependence of CLC-5 By Modifications of Two Conserved Charged Residues**

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The endosomal Cl<sup>-</sup>/H<sup>+</sup> antiporter, CLC-5, is inhibited by low extracellular pH. This could be caused by the saturation of a titratable residue which is directly involved in proton translocation towards the extracellular solution; alternatively, the reduction of currents at low pH could reflect an unspecific electrostatic effect of some titratable residue on proton and / or chloride movement within the external vestibule. To gain insight into the mechanism of the pH dependence we investigated the possible involvement of two highly conserved charged residues (D76 and K210) which are located in the extracellular vestibule. Mutants D76G and D76H drastically altered pH dependence. Preliminary experiments indicated that also K210C slightly altered the pH dependence. Interestingly, the K210C mutant could be modified by pCMBS (p-chloromercuriphenylsulfonic acid): application of 100 μM pCMBS led to a partial and irreversible inhibition of K210C mediated currents (but not WT CLC-5 currents). Our results favor the idea that neither D76 nor K210 are directly involved in proton translocation. However, further experiments, exploiting the possibility to modify K210C with various cysteine modifying reagents, are necessary to draw such a firm conclusion.

**1663-Pos****Role of the Q-R Linker in H<sup>+</sup>/Cl<sup>-</sup> Coupling in a CLC Exchanger**

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Members of the CLC family function as Cl<sup>-</sup> channels or as H<sup>+</sup>/Cl<sup>-</sup> exchangers. Despite this mechanistic divide the two CLC sub-classes share many functional and structural traits. CLC-ec1, a transporter, can be transformed into a Cl<sup>-</sup>-selective pore by simultaneously removing the intra- and extra-cellular gates. This entailed mutating two residues, E148 and Y445, which are conserved in both CLC channels and transporters. While the extracellular gate regulates channel gating, the intracellular one does not. We hypothesized that in the channels the intracellular gate's position is shifted so that it does not regulate ion passage anymore.

The linker connecting helices R and Q is 1-2 amino-acids shorter in the channels than in the transporters. We investigated whether shortening this linker disrupts the intracellular gate of CLC-ec1 while preserving Y445. To this end we deleted two residues, G441 and K442, in the Q-R linker. This deletion, ΔGK, functionally mirrors the gate-removed Y445A mutant: H<sup>+</sup> transport is nearly abolished with slight effects on Cl<sup>-</sup> transport. We then tested the effects of single-residue deletions. The ΔG mutant is similar to the WT while the ΔK deletion impairs H<sup>+</sup> transport like the ΔGK deletion. K442 however is not involved in H<sup>+</sup> transport: Alanine or Methionine substitutions have no significant effects.

Lastly, we incorporated in CLC-ec1 increasing portions of the CLC-0 channel. Introduction of the CLC-0 Q-R linker into CLC-ec1 recapitulates the functional effects of the ΔGK and ΔK mutations. Introduction into CLC-ec1 of up to the full helix R from CLC-0 led to no further effects on H<sup>+</sup> transport.

In conclusion we found that the length of the Q-R linker is critical for H<sup>+</sup>/Cl<sup>-</sup> coupling in CLC-ec1 while the chemical properties of the residues in the linker do not seem to matter.

**1664-Pos****Proton Block of Human CLC-5 Cl<sup>-</sup>/H<sup>+</sup> Exchanger**

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CLC-5 is a human Cl<sup>-</sup>/H<sup>+</sup> exchanger of the CLC family. It is expressed in intracellular compartments such as endosomes, where it regulates their acidification and is indirectly involved in the re-uptake of small molecular weight proteins in renal proximal tubule. Mutations in the CLC-5 gene lead to Dent's disease, a human disorder characterized by low molecular-mass proteinuria, kidney stones and renal failure. CLC-5 currents are strongly outwardly rectifying suggesting that the rates in the transport cycle are highly asymmetrical so that Cl<sup>-</sup> influx and H<sup>+</sup> efflux is permitted while the converse is not. Additionally CLC-5 currents are strongly inhibited by low external pH. It has been proposed that the rectification arises from the voltage dependence of a H<sup>+</sup> transport step and that the pH dependence is a result of a reduction in the total driving force for turnover. We studied the extracellular pH dependence of CLC-5 currents between pH 9 and 4.5. We found that the pH dependence is not solely due to changes in the driving force. Rather, we found that the degree of current inhibition is voltage dependent: at less positive potentials H<sup>+</sup> inhibition becomes stronger. This is consistent with a direct modulation of CLC-5 activity by H<sup>+</sup> at a single site located ~70% deep into the electric field. We propose that

this site is E211, the extracellular gating glutamate. In the presence of extracellular SCN<sup>-</sup> H<sup>+</sup> transport is abolished but both rectification and H<sup>+</sup> inhibition remain largely unaltered. This suggests that H<sup>+</sup> transport determines neither the pH dependence nor rectification. We propose that the extracellular pH inhibition of CLC-5 currents arises from H<sup>+</sup> trying to drive the transport cycle in the non-permissive direction. In other words, inhibition of the CLC-5 currents by extracellular H<sup>+</sup> is a consequence of its rectification.

**1665-Pos****A Monomeric Variant of the CLC-ec1 Transporter**

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CLC-ec1, a prototypical Cl<sup>-</sup>/H<sup>+</sup> exchanger of the CLC family, is a homodimer in which each subunit is thought to act as a fully functional transporter. Why then is this protein a dimer and what are the factors driving dimer formation? In this study, we address these questions by destabilizing the CLC-ec1 subunit-subunit interface. We substituted tryptophan residues to create steric mismatches at the interface that would also favor lipid interactions. A single mutation, I422W, was sufficient to destabilize the CLC-ec1 complex resulting in a shift from dimer to monomer position on gel-filtration chromatography. Glutaraldehyde cross-linking of I422W in both detergent and phosphatidyl-choline/phosphatidyl-glycerol liposomes showed that the mutant remained a monomer compared to the wild-type protein; however dimer formation was observable at high concentrations. The I422W mutant was functional as measured by 36Cl<sup>-</sup> uptake, passive Cl<sup>-</sup> efflux and Cl<sup>-</sup> driven H<sup>+</sup> pumping in *E. coli* phospholipids. A Poisson-counting method demonstrated that I422W re-dimerizes in these phosphatidyl-ethanolamine-rich liposomes, even at very low protein/lipid ratio. Altogether, these findings suggest that we have purified an isolated monomeric variant of CLC-ec1 that demonstrates shifted dimerization energetics depending on the lipid environment. We continue to search for mutations that will more severely destabilize the dimer, in attempts to obtain a transport-competent, monomeric CLC exchanger.

**1666-Pos****Analysis of the CLC-1 ATP Binding Site by Structure-Guided Mutagenesis**

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The CLC-1 chloride channel is abundantly expressed on the cell membrane of skeletal muscles, and thus plays an important role in controlling the muscle excitability. We previously showed that ATP can modulate the common gating of CLC-1, presumably by binding to a potential binding site at the C-terminal cytoplasmic region of the channel. The X-ray crystallographic study by others revealed the structure of the ATP-binding site, and two residues, Y617 and D727, were identified to be important for the ATP binding. Based on the CLC-5 C-terminal structure, we identify that besides V634 and E865 of CLC-1 (corresponding to Y617 and D727 of CLC-5), V613 and E860 are also important for the ATP modulation of the CLC-1 common gating. Mutating V634 to either alanine or aspartate abolishes the ATP modulation effect. Surprisingly, mutations of V634 to aromatic amino acids increase the ATP affinity: V634F and V634Y mutations decrease the apparent K<sub>1/2</sub> by ~30- and ~13-fold, respectively. Mutations of E865 to alanine and serine abolish the ATP modulation, while a charge-conserved mutation, E865D, greatly decreases the efficacy of the ATP effect—the maximal shift of the V<sub>1/2</sub> of the common gate P-V curve is ~10-20 % of that in the WT channel. Mutations of V860 and V613 to various amino acids also greatly reduce the efficacy of the ATP modulation. Double mutant cycle analyses show that V634 and V613 are strongly energetically coupled, with a coupling coefficient (Ω) of ~25, while V634 and E865 may be only weakly coupled with a Ω value of ~1.6. These results support the idea that ATP likely binds directly to the C-terminus of CLC-1 to modulate the common gating of the channel.

**1667-Pos****Substrate-Driven Conformational Changes in CLC-ec1 Observed by Fluorine NMR**

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The CLC "Cl<sup>-</sup> channel" family consists of both Cl<sup>-</sup>/H<sup>+</sup> antiporters and Cl<sup>-</sup> channels. This scenario presents a unique opportunity to investigate the molecular similarities and differences underlying these mechanisms. Although gating in CLC channels is known to involve large, cooperative conformational changes between protein subunits, it has been hypothesized that conformational changes in the antiporters may be confined to small movements localized near the Cl<sup>-</sup> permeation pathway. However, to date few studies have directly addressed this issue, and therefore little is known about the molecular movements that underlie CLC-mediated antiport. The crystal structure of the *E. coli* antiporter CLC-ec1 provides an invaluable molecular framework for such studies,