a buffer molecule, ΔH_{buf}. By measuring the enthalpy in several buffers we can determine the number of H^+ binding or unbinding from CLC-ec1 in response to Cl^- binding. We found that for WT CLC-ec1 n~0.5, suggesting that for every 2 Cl^- binding 1 H^+ is released. This is in agreement with the transport stoichiometry of CLC-ec1. This binding coupling is ablated by mutations that inhibit H^+ transport. Neutralization of either of the two H^+-accepting glutamates of CLC-ec1 leads to impairment in Cl^--coupled H^+ release.

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Mutation on the External Gate Changes the pH-dependency of a CLC Cl^-/H^+ Exchanger

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The CLC-ec1 is a bacterial homologue of the transporter-type CLC subclass that catalyzes transmembrane exchange of Cl^- and H^+. Glu_{ex} (E148 in CLC-ec1) is the pH-dependent external gate of both Cl^- and H^+ transport. The central tyrosine, Y445 (Tyr_{cen}) coordinates the central Cl^- ion and acts as the internal gate of the Cl^- pathway. Glu_{in} (E203) is remotely located to the internal Cl^- gate and acts as the internal proton-transfer residue that delivers protons from the internal aqueous solution to the protein interior. Wild type CLC-ec1 has the pH-dependent Cl- flux: as the pH increased, the transport rate of Cl- is decreased. It has been shown that mutations on the Glu_{ex} lead to the loss of pH-dependency of Cl^- flux while on the Glu_{in} retain relatively intact pH-dependency. We therefore examined mutations of Glu_{ex} and their functional consequences on the pH-dependency. Mutations with neutral amino acids, A or Q (E148A or E148Q) completely lose H^+ movement as well as pH-dependency of Cl^- transport. E148H mutant also fail to transport H^+, but strikingly reverses pH-dependent transport of Cl^-, as does the doubly mutated transporter, E148H/E203H. These data support the idea that the pH-dependency is mainly came from the Glu_{ex} not Glu_{in}. Currently, we are investigating the effect of other mutants having various ranges of pKa value and their structural and functional consequences.

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Measuring ClC-Transporters on Solid Supported Membranes

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Members of the family of ClC-chloride channels and transporters have received increasing attention in the last years because of their important physiological functions and their implication in pathogenesis. Some are important targets for drug discovery while others (e.g. ClC-7) are still poorly investigated due to the lack of suitable electrophysiological methods.

Recently, electrophysiological measurements based on solid supported membranes (SSM) have been used for the functional characterization of ion pumps and transporters. In this technique proteoliposomes, membrane vesicles or membrane fragments are adsorbed to a SSM and are activated by rapid substrate concentration jump. Then charge translocation is measured via capacitive coupling to the supporting membrane.

SSM-based electrophysiology is extremely useful in cases where conventional electrophysiology cannot be applied. Apart from a few rare exceptions bacterial transporters cannot be investigated using voltage clamp or patch clamp methods and also mammalian transporters predominantly expressed in intracellular compartments are not accessible for standard electrophysiology.

Here we apply SSM-based techniques to two proteins of the ClC-family which function as H+/Cl- exchangers: the bacterial ClC-ec and the putative transporter ClC-7 from lysosomes. ClC-ec was purified from E. coli and reconstituted into liposomes. The proteoliposomes were adsorbed to the SSM and transient currents were measured after activation of the protein with a Cl- concentration jump. This allows time resolved measurements of charge translocation and kinetic analysis of the transport mechanism. ClC-7 was investigated using a stable expressing CHO cell line. Cell membranes were prepared and adsorbed to the SSM. Also ClC-7 was activated using a Cl- concentration jump and transient currents were recorded. This allows the first electrophysiological characterization of ClC-7 and its functional analysis.

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Functional Characterization Of Clc-5 Mutations Associated With Dent's Disease

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The Cl^-/H^+ antiporter ClC-5 has been linked to Dent's disease, an X-linked renal disease associated with low molecular weight proteinuria, hypercalciuria and nephrolithiasis. ClC-5 is expressed on early endosomes of proximal tubule cells, where it plays a critical role in endosomal function. The impact of Dent's disease-causing mutations on ClC-5 function has not been yet fully investigated. Here, we have analysed an unpublished mutation K115R and three published mutations, Y272C, N340K and K546E in terms of electrical activity and trafficking at the plasma membrane in *Xenopus leavis* oocytes. A construct carrying an extracellular HA epitope (kindly provided by T. J. Jentsch, MDC/FMP, Berlin) that does not alter the ClC-5 wild-type (WT) currents allowed us to evaluate surface expression of the different ClC-5 using a chemiluminescence test. The currents were measured by two-electrode voltage-clamp. The mutant K115R induced a reduction of 68 ± 1.9% of WT ClC-5 currents ($p<0.001$, $n=17$). Currents recorded with Y272C ($n=6$), N340K ($n=7$) and K546E ($n=6$) mutants were not significantly different from non-injected oocytes. The loss of currents for the mutants N340K and K546E correlated well with a loss of surface expression : chemiluminescence signals were not significantly different from those observed in non-injected oocytes ($p<0.001$, $n=6$). We found no significant difference between surface expression of K115R ($n=5$), Y272C ($n=4$) and WT ClC-5. In conclusion, N340K and K546E mutants have a defective targeting to the oocyte plasma membrane, and K115R and Y272C a reduced electrical activity. Further studies should investigate whether the targeting to early endosomes is faulty in the case of the first type of mutations and how the regulation or the conduction pathway are involved in the case of the second type of mutations.

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Binding Sequence And Coupling Of H+ And Na+ Ions In The Glutamate Transporter (glt)

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Substrate transport in GlT is catalyzed by co-transport of three Na^+ (Na1, Na2 and Na3) and one H^+, and counter-transport of one K^+. The crystal structure of GlT, however, provides information only about the position of the substrate and two Na^+ ions. In order to investigate the transport cycle in GlT, therefore, the first step is to identify the binding sites for the H^+ and for the third Na^+ ion. Careful examination of the structure in the context of the possible transport pathway indicates that there are only two titratable acidic residues (Asp312 and Asp405) that might serve as the H^+ binding site during the transport cycle. We have performed a set of MD simulations (30 ns each) with different combinations of substrate and protonation states of Asp405 and Asp312. Our simulations show that water has no access to Asp312, but can easily reach Asp405 in the apo state, suggesting that Asp405 is possibly the first H^+ binding site. The access of Asp312 to the extracellular solution is blocked by an H-bond network between Asn310, Asn401, and Asp405. Interestingly, protonation of Asp405 results in disruption of this network allowing access of water to Asp312. In search for the third Na^+ binding site, a water molecule near Asp312 (a putative Na^+ binding site) was randomly replaced by a Na^+ at the beginning of four independent simulations. The placed Na^+ was observed to move into and be stabilized by a binding site formed by Asp312, Thr92, Asn310 and Ser93. Based on these results, we propose that H^+ binding precedes that of Na3, as protonation of Asp405 is required for hydration of Asp312 and providing access for Na^+ binding.

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Exploring the Gating Mechanism of the Glutamate Transporter GltPh

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Glutamate acts as the primary neurotransmitter in the mammalian central nervous system. Clearance of this neurotransmitter from the synapse is accomplished by a family of glutamate transporters known as EAATs. These proteins move synaptic glutamate across the cell membrane into the cell against its concentration gradient. It has long been speculated that the mechanism for transport involves the movement of extracellular and intracellular gates, providing ''alternating access'' to a substrate binding site. Recently, the crystal structure of a related bacterial transporter, GltPh, was solved, revealing two helical hairpins (HP1 and HP2) which have been proposed to contribute to these gates. HP2 lies on the extracellular face of the transporter, and a number of studies have shown that this hairpin can adopt multiple conformations that either provide or restrict access to the substrate binding site. However, to date there is no structural information regarding the conformational changes that must occur within HP1 in order to provide the substrate a path to the intracellular solution. Here we use the technique of site-directed spin labeling (SDSL) electron paramagnetic resonance (EPR) spectroscopy to explore the structure of HP1 (residues 258-290) in purified GltPh reconstituted into proteoliposomes. This technique provides an opportunity to probe the dynamic structure of HP1 in a functional protein embedded in a physiological lipid environment.