Molecular aspects on voltage-sensor movement

Amir Broomand
To my beloved

Neda
ABSTRACT

Voltage-gated ion channels are fundamental for electrical signaling in living cells. They are composed of four subunits, each holding six transmembrane helices, S1-S6. Each subunit contains a voltage-sensor domain, S1-S4, and a pore domain, S5-S6. S4 contains several positively charged amino-acid residues and moves in response to changes in membrane voltage. This movement controls the opening and closing of the channel. The structure of the pore domain is solved and demonstrates principles of channel selectivity. The molecular mechanism of how the voltage sensor regulates the opening of the channel is still under discussion. Several models have been discussed. One of the models is the paddle model where S3b and S4 move together. The second one is the helical-twist where S4 makes a small rotation in order for the channel to open. The third one is the helical-screw model where S4 twists around its axis and moves diagonally towards the extracellular side of the channel.

The aim of this PhD project was to study the molecular movement of the voltage sensor in the depolarization-activated Shaker K channel. Cloned channels were expressed in *Xenopus laevis* oocytes, and investigated with several electrophysiological techniques.

1. We show that S4 moves in relation to both S3b and S5. The formation of some disulfide bonds between S4 and neighboring positions, in only the open state, shows that the paddle model cannot be correct. Furthermore, electrostatic and steric effects of residues in S3b suggest that S3b is tilted, with the intracellular part close to S4.

2. We show that the relatively Mg-sensitive Shaker K channel is changed into the less Mg-sensitive Kv2.1 K channel with respect to its sensitivity to extracellularly applied Mg$^{2+}$ by changing the charge of three extracellularly positioned amino acid residues. One of the residues, F425C, mediates its effect through the neighboring residue K427.

3. We show that oxaliplatin, an anti-cancer drug, has no effect on the Shaker K channel. It has been suggested that a negatively charged monochloro complex of oxaliplatin is the active substance, and also causes the neurotoxic side effects. Neither this complex shows any effect on the channel.

Our experiments point towards the helical-screw model. The other models for voltage-sensor movements are incompatible with the results in this study.
LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. Amir Broomand, Roope Männikkö, H. Peter Larsson and Fredrik Elinder
   *Molecular movement of the voltage sensor in a K channel.*

II. Amir Broomand and Fredrik Elinder
    *Disintegration of the voltage-sensor paddle during potassium-channel gating.*
    Manuscript

III. Amir Broomand, Fredrik Österberg, Tara Wardi, and Fredrik Elinder
     *Electrostatic domino effect in the Shaker K channel turret*

IV. Amir Broomand, Elin Jerremalm, Jeffrey Yachnin, Hans Ehrsson, Fredrik Elinder
    *Oxaliplatin neurotoxicity – no general ion channel surface-charge effect*
    Manuscript
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INTRODUCTION

Ion channels are found in all organisms from bacteria to humans and have very diverse functions in controlling physiological events such as hormone secretion, heart beat, muscle contraction, and blood pressure (Hille, 2001). Voltage-gated ion channels are essential for the propagation of nervous impulses (Hodgkin, 1964) and signaling in the nervous system. Sodium and potassium ions passing through the open channels transmit electrical signals which underlie all nervous functions and consequently human action. In order to make use of electrical signals, parts of the voltage-gated ion channels have developed to detect the electrical changes in the membrane voltage and control the opening and closing of the channel. These electric-detecting parts are known as voltage sensors. However, the molecular structure and its exact function is still unclear and under relentless discussions. The present thesis focuses on the movement of the voltage sensor in the Drosophila melanogaster Shaker H4 voltage-gated K channel (Kamb et al., 1987; Tempel et al., 1987; Pongs et al., 1988), here after referred to as the Shaker K channel.

Voltage-gated K ion channels

Voltage-gated K ion channels are proteins imbedded in the bilayer of cell membranes. They contain about 2000 amino-acid residues and belong to the superfamily of voltage-gated-like ion channels to which also Na and Ca channels belong, all with similar molecular structure (Strong et al., 1993; Milkman, 1994; Hille, 2001; Yu and Catterall, 2004). They consist of four subunits packed together in a square-like structure with the ion permeable pore in the center (Figure 1a). Each subunit consists of six transmembrane (6TM) helices. The helices S1 to S4 form the voltage-sensor domain (VSD) and helices S5 and S6 form the pore domain together with the pore-forming loop which stretches half way through the membrane (Figure 1b). The helix number 4 (S4) has positively charged amino acids at every third position and is the actual voltage sensor of the channel.

The pore domain

For the study of the pore domain of the ion channels the bacterial two-transmembrane helix (2TM) channel KcsA (Doyle et al., 1998) is commonly used as model. Each 2TM subunit has two helices and a pore loop which in similar fashion to a 6TM subunit, forms the pore domain when four KcsA subunits are joined together (Figure 1a). The amino-acid sequence of the pore domain of Shaker K and KcsA are homologous (Figure 1c), and the extracellular architecture of the channel has been shown to be similar (MacKinnon et al., 1998).

The inner transmembrane α-helix of KcsA, analogous to S6 of voltage-gated ion channels, lines the intracellular half of the ion permeating pore. The four inner helices from the four subunits form a bundle at the intracellular end that closes the KcsA channel pore (Figure 1d). The helices are somewhat tilted so that the pore widens to form a cavity above
Figure 1. a) A schematic drawing of the four domains showing a voltage-gated ion channel from above (extracellular part). The dashed diamond shows the four pore domains. b) A schematic drawing of the six transmembrane helices (S1 to S6) and the pore loop (P) of one subunit. VSD stands for Voltage-Sensor Domain. c) The amino acid sequence of Shaker K and KcsA channel. The gray areas denote very well conserved areas among most of the potassium channels. d) A schematic x-ray crystallographic structure of KcsA channel (PDB code 1BL8). For clarity only two of the subunits are shown. The pore loop is shown in white and the inner and outer helices are shown in gray. The selectivity filter which is at the extracellular end of the channel is marked by a white dashed ellipse. The main activation gate is marked by a dashed rectangle.
the bundle crossing. The outer helices, analogous to S5, are located lateral to the pore, facing the lipid bilayer. The pore-loop extends from the extracellular end of the outer helix, turns into the lipid bilayer, continues halfway across as an α-helix (the pore helix) and makes a loop all the way to the extracellular end of the inner helix.

The selectivity filter

The selectivity filter is located in the extracellular part of the pore and is ideally organized for efficient selectivity of potassium ions through the pore (Figure 1d). The selectivity filter contains a signature sequence, GYG (Heginbotham et al., 1992), where the backbone carbonyl oxygens of this sequence together with a side chain oxygen from a nearby threonine are organized in such way, that they provide essentially an equal environment for passage of potassium ions as if the ions were in the hydrated state. This is the suggested mechanism for the ion selectivity of the potassium channels. The precise arrangement of the oxygens and the rigid conformation of the selectivity filter itself minimize the energetic loss when a potassium ion sheds its water molecules as it enters the selectivity filter, while the smaller sodium ion would not be provided with any favorable environment when entering the filter (Doyle et al., 1998; Aqvist and Luzhkov, 2000; Morais-Cabral et al., 2001; Zhou et al., 2001). Small changes in the molecular arrangement of the selectivity filter can limit the ion permeation, and it has been suggested that the selectivity filter can function as an activation gate (Chapman et al., 1997; Zheng and Sigworth, 1998; Liu and Siegelbaum, 2000).

Activation and inactivation gates

The main gate of a voltage-gated ion channel is located at the intracellular part of the pore. The access of intracellularly applied channel blockers to their blocking site was shown to depend on the opening and closing of the channel (Armstrong, 1971). The gate of KcsA channels resides in the crossing of the inner helices (The bundle crossing, Figure 1d) of KcsA (Doyle et al., 1998), S6 in the case of Shaker K like channels. Perozo et al. (1999) showed that the greatest conformational changes in KcsA during opening and closing, take place at the intracellular end of the inner helices. Their result suggests that the inner helices move farther apart and twist while the selectivity filter remains rigid and unchanged during gating.

The KcsA structure represents a potassium ion channel in the closed state. To be able to study the open state of a potassium ion channel a Ca²⁺-gated potassium channel, MthK (Jiang et al., 2002a; Jiang et al., 2002b), was used. The sequences of the transmembrane segment of the KcsA and MthK channels are homologous. The difference between the two channels is that the intracellular part of the MthK structure is much wider compared to the structure of the KcsA, which means that the MthK channel is open at the intracellular part. At the pivot point for this opening, a conserved glycine can be found, which is known to act as a hinge. Therefore, at this point the inner helix of a potassium channel is able to kink which makes gating possible.
There are two separated inactivation mechanism in the Shaker K channel called N-type and C-type inactivation. The N-type inactivation will be discussed in the section about intracellular domains. The C-type inactivation in Shaker K like channels, also called the slow inactivation, has been shown to take place in the selectivity filter region, seen in Figure 2 (inactivated channel) as horizontal arrows (Hoshi et al., 1991; Lopez-Barneo et al., 1993). Studies suggest that removal of potassium ions in the selectivity filter leads to repulsion of carbonyl oxygens. The filter destabilizes and collapses, thereby accelerating the slow inactivation (Almers and Armstrong, 1980; Liu et al., 1996; Loboda et al., 2001; Zhou et al., 2001). It has also been suggested that a rotation in the linker between the pore and S6 leads to the collapsing of the pore (Larsson and Elinder, 2000).

**The voltage-sensor domain**

The voltage-gated ion channels open and close in response to changes in the transmembrane voltage. This voltage-dependent opening and closing is controlled by the voltage-sensor domain of each subunit in the channel (Figure 1b). The voltage sensor itself is the fourth transmembrane helix, S4, since it contains several (3 – 9) regularly spaced positively charged amino acids at every third position, separated by hydrophobic amino acid residues. The location of a positive charge in the membrane bilayer is energetically unfavorable and is therefore compensated with negatively charged amino acid residues in the transmembrane helices S2 and S3 (Papazian et al., 1995; Tiwari-Woodruff et al., 1997; Keynes and Elinder, 1999; Tiwari-Woodruff et al., 2000; Lecar et al., 2003). In the resting state, the voltage sensor is attracted to the negative cell interior (Figure 2), hence finding itself at a “down” position. As the transmembrane voltage is depolarized and the inside of the cell becomes more positive, the positive charges of the voltage sensor are repelled outward leading to the activation of the

![Figure 2. Molecular organization of the activation and inactivation of a voltage-gated ion channel. The horizontal black bar represents the bundle crossing (crossing of the inner helices). The ball and chain mechanism sits at the N terminal of each subunit. For clarity only one of the subunits holds the ball and chain and the other one the bundle crossing.](image-url)
channel. The repulsion of the voltage sensor moves three positive charges from the inside of the membrane to the outside (Schoppa et al., 1992). But in what manner does the voltage sensor actually move?

**Proposed models for voltage sensor movement**

A detailed description of the molecular movement of the voltage sensor is essential for understanding the function of the voltage-gated ion channels which can lead to an insight of how pharmacological compounds can affect the channel’s voltage sensitivity. The past two dominating models have been the helical-screw model (Figure 3a) (Catterall, 1986; Guy and Seetharamulu, 1986; Keynes and Elinder, 1999; Lecar et al., 2003) and the helical-twist model (Figure 3b) (Papazian and Bezanilla, 1997; Bezanilla, 2000). These two models have recently been challenged by the paddle model developed from KvAP (Figure 3c) (Jiang et al., 2003b).

In the presence of deep, water-filled crevices and a tilted S4, a simple twist of the voltage sensor α-helix would relocate three charges from the intracellular crevice of the channel to the extracellular crevice without any large-scale molecular movement (Figure 3b) (Papazian and Bezanilla, 1997; Bezanilla, 2000). The problem with this model is that the conserved arginines in the S4 has been shown to move 15-20 Å through the thickness of the membrane (Ruta et al., 2005). In 2003 a new model for the voltage sensor of all potassium channels, the so called paddle model, was presented (Jiang et al., 2003b). Here the C-terminus of S3, the S3b, and the S4 form a stable helix-loop-helix structure that lays parallel to the membrane plane at the intracellular side. The paddle is stabilized by hydrophobic and electric

![Figure 3](image)

**Figure 3.** The molecular models of the voltage sensor movement. In all of the cases three positive charges (not necessarily the same ones) are relocated from the intracellular to the extracellular side of the channel. -- denotes a negative membrane potential at which a voltage gated channel is in the hyperpolarized (down) state. +++ denotes a positive membrane potential at which a channel is in the depolarized (up) state.
interactions and was suggested to move as a single unit (Figure 3c). Today, it is an accepted fact that each S4 carries three positive charges from the intracellular part of the channel to the extracellular part in order for the channel to open (Figure 3). At least, in the Shaker K channel the amino acid sequence (paper II, Figure 2b) shows that when the positive charges are subtracted from the negative ones, the net charge of a possible paddle will only be 1+. This means that each paddle in the Shaker K channel would only carry one single positive charge as the channel opens. This is contradictory to the accepted fact.

In the helical-screw motion, the S4 moves both in a helical-twist motion (as in a corkscrew) and an upward motion into the extracellular part of the channel (Figure 3a). We will investigate this model further and try to detect which model is best fitted with our results.

**Gating currents**

The movement of the charges of the voltage sensor generates a current by itself which has come to be called the gating currents. These currents can be measured when the ionic currents are blocked (Armstrong and Bezanilla, 1973; Keynes and Rojas, 1973). The gating currents are often fast and precede the channel opening as predicted by Hodgkin and Huxley (1952). They give valuable information regarding the movement of the voltage sensor. Neutralization of any of the first four positive charges in S4 of the Shaker K channel decreases the gating charge (Aggarwal and MacKinnon, 1996; Seoh et al., 1996).

**The intracellular domains**

A small change in the membrane voltage triggers changes in the molecular machinery of a voltage-gated channel, beginning with the movement of the voltage sensor which ultimately leads to the opening the channel. How are the different channel units connected to form a functional voltage-gated ion channel?

**The N-type inactivation**

The N-type inactivation, also called the fast inactivation, makes use of a so called ball-and-chain mechanism, seen in Figure 2 as a light gray circle linked to a subunit. The model for ball-and-chain predicts that deletion of the ball and the chain should eliminate the fast inactivation, and reperfusion of the missing piece into the cytoplasm might restore inactivation. These predictions were confirmed by studies on the Shaker K channel (Hoshi et al., 1990; Zagotta et al., 1990). The N-terminal inactivation ball has also been shown to prevent the return of the voltage sensor from the activated state. This is called charge immobilization, where the Off gating charge is not equal to the On gating charge (Armstrong and Bezanilla, 1977; Bezanilla et al., 1991). When the N-terminal ball is removed, the Off gating and On gating charges are equal.

**The voltage sensor linker**

The voltage sensor is linked to the pore domain with a peptide linker between S4 and S5 (Figure 2). This linker has been suggested to be one of the major factors in the coupling of the
The movement of the voltage sensor mechanically pulls the linker thereby exerting a tugging force on the intracellular ends of the S5 and S6 segments.

**Tetramerization (T1) domain**

The T1 domain is a 130 residue long part of each subunit, between the ball and chain and S1. This N-terminus part of the channel participates in the tetramerization of the subunits but forms neither a gate nor a necessary part of the conducting pore since the channel function normally without the T1 domain (Hille, 2001). Similar to the rest of the subunits, the T1 domains are identical (Kreusch et al., 1998). Since this domain is directly placed below the pore entryway to the cytoplasm, the potassium ions must flow through side portals in order for the transmembrane pore and the cytoplasm to communicate (Gulbis et al., 2000; Kobertz et al., 2000; Sokolova et al., 2001). These portals are large enough to permit the entry of the N-terminal (ball-and-chain) inactivation gate (Hoshi et al., 1990; Zagotta et al., 1990).
AIM

The general aim of this work has been to study the movement of the voltage sensor of the Shaker K channel. The specific aims are:

- How does the voltage sensor move in relation to the pore domain?
- How does the voltage sensor move in relation to other parts of the voltage sensor domain?
- How is the voltage sensor affected by extracellular metal ions?
- How is the voltage sensor affected by a platinum-based anti-cancer drug?
METHOD

Molecular biology

The experiments were performed with voltage-gated Shaker H4 potassium channels (Kamb et al., 1987) made incapable of fast inactivation by the Δ(6–46) deletion (Hoshi et al., 1990). Mutations were introduced with QuickChange Kit (Stratagene) and verified by sequencing with DynaKit (Amersham Pharmacia Biotech Inc.). cRNA was transcribed from purified DNA using T7 mMachine Kit (Ambion Inc.). The integrity and the amount (ng/µL) of the cRNA was measured by ND-1000 Spectrophotometer, Nanodrop Technologies.

Xenopus laevis and its oocyte

*Xenopus laevis*, also called African clawed frog, is found naturally in southern Africa. The species can be found in the northern part of South Africa, Zambia, and Botswana and also in the mid west of Africa in countries such as Cameroon, Nigeria, and Congo Brazzaville (Evans et al., 2004). *X. laevis* lives in pond and streams usually devoid of higher plant vegetation and covered in green algae. It is almost totally aquatic, only leaving the water when forced to migrate (Garvey, 2000).

The females weigh about 200 g and are about 10 to 12 cm long. The males are smaller and weigh about 60 g and are 5 to 6 cm long (Figure 4a). Other than the size the females can be distinguished from the males by a small cloacal extension which males lack, at the end of the abdomen (Garvey, 2000).

*Xenopus laevis* oocytes have been the classic expression system for ion channel genes (Miledi et al., 1982; Shih et al., 1998) since they are large and easy to handle. They are classified in six stages, denoted I – VI, each stage referring to a specific period of development with stage VI being the highest (Figure 4b).

The oocytes have few endogenous channels which seldom interfere with the exogenously expressed channels as they are normally activated at higher voltages (>60 mV).
and carry small currents compared to the exogenously expressed channels (-80 mV – 50 mV). The channels expressed in oocytes display similar currents as the ones expressed in mammalian systems indicating that the properties of the expressed channels are not affected by endogenous factors in the oocytes. In the present studies all injected oocytes displaying suspect endogenous currents were discarded. All the oocytes used for injection were healthy mature oocytes.

Surgery and injection

In order to get oocytes, a female *Xenopus laevis* was anaesthetized with 1.4 g/L Ethyl 3-aminebenzoate methanesulfonate salt (tricaine, Sigma-Aldrich). A batch of oocytes was removed and the surgery wound stitched together. The oocytes were rinsed in OR-2 (Table 1) and incubated in OR-2 containing Liberase Blendzyme 3 (7 U, Roche Diagnostics) for about 1.5 to 2 h to separate them from the ovary sack. Healthy mature oocytes (Figure 4b) were singled out and incubated in Modified Barth’s Solution (MBS, Table 1) containing 2.5 mM pyruvate and 2.5 µg/µl penicillin-streptomycin solution (Sigma-Aldrich) over night before injection.

50 nl cRNA (20-500 pg/cell) coding for the channel was injected into *Xenopus laevis* oocytes using a nanoject injector (Drummond Scientific Co.). The oocytes were incubated in MBS containing pyruvate and antibiotics until subjected to electrophysiological experiments (usually 3-5 days after injection). In the case of double cysteine mutations, 0.5 mM dithiothreitol (DTT) was added to the MBS to prevent the cysteines from forming a disulfide bridge.

Electrophysiology

The electrophysiological properties of the oocyte-expressed channels were studied by two-electrode voltage clamp. This method allows continuous rinse of extracellular solutions over the oocytes. The effect of different extracellular channel modulators is easily studied in this set-up. Two microelectrodes were inserted into the oocyte: one is used to measure the voltage difference between the cell interior and the extracellular reference electrode, while the second one is used as a current injector.

In paper IV, in addition to two electrode voltage clamp, the cut-open voltage clamp was used. This method is relatively fast and has a low current noise (Taglialatela et al., 1992). Another advantage is that the intracellular side of an oocyte can be controlled. For measurement of the currents, the oocyte is placed between three chambers: (1) the recording chamber where a microelectrode is inserted into the domus of the oocyte and keeps the membrane under clamp; (2) the middle chamber that acts as a guard shield; and (3) the bottom chamber which injects current. Before recording, the bottom of the oocyte was permeabilized by 0.1 % saponine.
For the measurement of the currents in all of the experiments, borosilicate (Harvard apparatus Ltd, GC150-10) microelectrodes were pulled (Narishige PP-830 puller) and filled with 3 M KCl solution. The resistance of the microelectrodes varied between 0.5 – 2.0 MΩ. The currents were recorded by CA-1 amplifier (Dagan corp.). Leak resistance and analogue capacitance compensations were used and currents were low-pass filtered at 5 kHz. The extracellular solution used was 1K (Table 1). In the case of Cl-free intracellular solution in the bottom chamber for cut-open measurements, Cl-free 100Kin (Table 2) solution was used. This is especially useful for recordings at very negative voltages, where Cl⁻ ions moving from the inside of the channel to the outside can disturb the recordings. In the recording chamber Cl-free 100Kout (Table 2) was used. All experiments were done at room temperature (20 – 23 °C).

The software pCLAMP from Axon instruments was used for recording the currents and voltages. For analysis of the recordings, Clampfit (Axon Instruments) and GraphPad (GraphPad Software, Inc.) was used.

**Cysteine modification**

Cysteine has a special status in protein research due to availability of a wide variety of cysteine-specific reagents. To use a cysteine mutation as a research tool, first a desired position is mutated. Then cysteine-specific reagents are allowed to react with the cysteine, changing the charge and the size and also adding fluorescent groups or other functionalities (Hille, 2001).

Electrophysiologically, a channel can be studied if the binding of a reagent to a cysteine in the channel changes the characteristics of this channel. The modification rate contains information about the local environment of the cysteine. For instance a fast modification could mean an exposed cysteine and a slow modification a buried cysteine.

### Table 1. The concentration of the chemicals in the solutions used for surgery and electrophysiology. All concentrations in mM.

<table>
<thead>
<tr>
<th></th>
<th>NaCl</th>
<th>KCl</th>
<th>MgCl₂</th>
<th>CaCl₂</th>
<th>Ca(NO₃)₂</th>
<th>MgSO₄</th>
<th>NaHCO₃</th>
<th>HEPES</th>
</tr>
</thead>
<tbody>
<tr>
<td>OR-2</td>
<td>82.5</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>MBS</td>
<td>88²</td>
<td>1</td>
<td>-</td>
<td>0.41</td>
<td>0.33</td>
<td>0.82</td>
<td>2.4</td>
<td>15</td>
</tr>
<tr>
<td>1K</td>
<td>88²</td>
<td>1</td>
<td>0.8</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>15</td>
</tr>
</tbody>
</table>

All of the solutions were adjusted to pH 7.4 by NaOH

² Added NaOH gives a final [Na⁺] of ~100 mM

### Table 2. The Cl-free solutions used for cut-open. All concentrations in mM.

<table>
<thead>
<tr>
<th></th>
<th>KOH</th>
<th>Methanesulfonic acid</th>
<th>HEPES</th>
<th>EGTA</th>
<th>CaCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl-free 100Kₙ</td>
<td>110</td>
<td>110</td>
<td>10</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>Cl-free 100Kₙ</td>
<td>107</td>
<td>107</td>
<td>10</td>
<td>-</td>
<td>2</td>
</tr>
</tbody>
</table>

All of the solutions were adjusted to pH 7.4 by KOH
In my case, I used the cysteine mutations to try to make bridges between two closely located positions (Figure 5a), usually 4 – 5 Å between the β-carbons (Careaga and Falke, 1992). To induce a disulfide-bond formation, 2 µM CuSO₄ and 100 µM Phenantroline was added to the 1K solution and to break the disulfide bond, 20 mM DTT was added. I also did cysteine-reactivity studies where I changed the charge of the cysteine mutation (Figure 5b). To add a single positive charge 100 µM [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET) was used. For a single negative charge 1mM sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES) was used. These chemicals were purchased from Toronto Research Chemicals Inc. In all of the cases the cysteine-modifiers were applied in the bath by a manually controlled gravity-driven perfusion system. To prevent degradation of MTSET and MTSES, they were freshly dissolved, just before the experiments, and kept on ice.

\[ \text{2 Cysteines} \quad >\text{C – CH}_2 – \text{SH} + \text{HS – } \mu \text{HC – C} < \]

\[ \text{Cu/ Phenantroline} \quad \uparrow \quad \text{DTT} \]

\[ >\text{C – CH}_2 – \text{S – S – 2HC – C} < \]

\[ \text{Cysteine + MTSES} \quad >\text{C – CH}_2 – \text{S – S – CH}_2 – \text{CH}_2 – \text{SO}_3^- \]

\[ \text{Cysteine + MTSET} \quad >\text{C – CH}_2 – \text{S – S – CH}_2 – \text{CH}_2 – \text{N}^+(\text{CH}_3)_3 \]

**Figure 5.** a) Schematic structure of formation and breakage of disulfide bond. b) Schematic structure of cysteine residues with attached MTS reagents. For details see text.

**Oxaliplatin**

Oxaliplatin (Figure 6) is a platinum-based anti-cancer drug which, in combination with 5-fluorouracil, is used for the treatment of colorectal cancer (Judson and Kelland, 2000). The proposed action of this drug is the formation of platinum-DNA adducts (Mani et al., 2002). Oxaliplatin has been shown to be reactive towards sulfur containing compounds, such as cysteine. The suggested mechanism of the reaction is a direct attack of the sulfur on the platinum (Mani et al., 2002). At physiologic temperature and chloride concentration, the negatively charged monochloro complex is formed and constitutes about 10% of intact oxaliplatin within 30 minutes. Subsequently, the dichloro complex is formed (Jerremalm et al., 2004).
There have been reports of neurotoxic effect from treatment by oxaliplatin (Wilson et al., 2002). These effects are acute, and appear usually within hours. This is why it is believed that the formation of the monochloro complex, which also is fast, is the reason for the neurotoxicity effects.

**Figure 6.** The molecular structure of oxaliplatin (left), its monochloro complex (middle), and dichloro complex (right).
RESULTS AND DISCUSSION

In the present thesis, I have focused on the molecular movement of the voltage sensor of the Shaker K channel. I have also investigated the effects of charged compounds applied to the extracellular side of the channel and their electrostatic effects on the voltage sensor. The first part is largely about double-cysteine mutations, one cysteine positioned in the S4 helix and one positioned, either in S3b or in the extracellular part of S5. The focus is on whether any of the double mutations can form a disulfide bond. These contact surfaces will elucidate structural constrains for the voltage-sensor movement. Furthermore, the effects of positive and negative charges in the mentioned positions, on the voltage sensor, will be studied. The second part is about how positively and negatively charged ions affect the voltage sensitivity of the channel.

Voltage-sensor movement (Papers I and II)

In papers I and II, I tried to distinguish between the three most used models for the movement of the voltage sensor: the helical-screw model, the paddle model, and the helical-twist model (Figure 3). Two specific questions were addressed. Paper I: Is the extracellular tip of S4 distant to the pore domain as suggested by the KvAP structure (Jiang et al., 2003a), or is it close to the pore domain as suggested by Elinder and collaborators (Elinder and Arhem, 1999; Elinder et al., 2001a; Elinder et al., 2001b). Paper II: Is S4 fixed in relation to S3b as suggested in the paddle model (Jiang et al., 2003a; Jiang et al., 2003b; Long et al., 2005a; Long et al., 2005b), or is it moving as suggested by the helical-screw model (Catterall, 1986; Guy and Seetharamulu, 1986; Keynes and Elinder, 1999; Lecar et al., 2003).

In paper I we studied six double-cysteine mutations, and attempted to form disulfide bonds between the extracellular end of the S4 and the extracellular end of S5. The results show that three of these pairs form disulfide bonds. Specifically distinguishable is the bond formation between amino acid residues 362C and 416C, where the channel currents almost totally disappeared when a disulfide bond was formed (Figure 7a). This disulfide-bond formation is state dependent and is formed much faster in the open state of the channel, when all of the S4:s are in the upper position, than in the closed state (Figure 7b). In Figure 7b, the disulfide bond formation is seen as a decrease of the current. The disulfide bond was formed spontaneously when DTT was removed from the extracellular solution, or, much faster, when Cu/Phenantroline was added to the extracellular solution. These experiments clearly demonstrate that the voltage sensor S4 is close to S5 in the pore domain in the open state. This finding is not compatible with the structural model based on the first X-ray data from KvAP (Jiang et al., 2003a). However, it fits well with the earlier electrostatic experiments (Elinder and Arhem, 1999; Elinder and Arhem, 2003), the later X-ray structures (Long et al., 2005a), and other disulfide-bond experiments (Aziz et al., 2002; Laine et al., 2003).
If the disulfide bond is formed in the open state, why does the current decrease at all? The answer is that the current reduction is caused by slow (C-type) inactivation. Although our channel is deprived of its fast inactivation, the slow inactivation still occurs making the current to decrease. The disulfide-bond formation between S4 and S5 prevents the voltage sensors to move back. This means that the channel cannot recover from the inactivated state and therefore cannot reopen. Does the disulfide bond occur within one subunit or between two subunits? In paper I we also show that a disulfide bond is formed when single-cysteine mutations 359C and 416C are co-injected (Figure 7c). This shows that the bond is formed between two adjacent subunits and not within one subunit. This finding was later confirmed by an X-ray crystallographic structure (Long et al., 2005a).

In paper II, we show that the intracellular end of S3b, but not the extracellular end, is close to S4 suggesting that S3b is tilted compared to S4. Here all single cysteine mutations from positions 323 to 333 were studied. The strategy was to change the charge of each residue by adding either the positively charged cysteine-specific reagent MTSET or the negatively charged cysteine-specific reagent MTSES. MTSET shifts the channel’s voltage dependence in positive direction and in MTSES shifts it in negative direction. The closer to S4, the larger should the effect be. This experimental technique has been evaluated and successfully used before (Elinder et al., 2001a). However, if the applied reagent is attached very close to the voltage sensor there will be a sterical component in addition to the electrostatic component. The sizes of the two components can easily be calculated (see paper II for more details). The steric effect is equal to half of the summed shift values for MTSET and MTSES. The rest is the electric effect.

All of the studied mutations were modified by MTSET/MTSES suggesting that all are, at least temporarily, exposed to the extracellular solution. Both the steric and electrostatic components showed a very clear pattern. The largest effects were found at the intracellular end of S3b and the smallest effects were seen at the extracellular end. The cysteine mutation

![Figure 7](image_url)
Figure 8. a) The steric effect (left) and the electrostatic effect on S4 by MTSET and MTSES. b) Disulfide bond formation in 325C/366C. Left: Voltage-clamp family in control solution. The voltage is between -80 mV and +50 mV in steps of 5 mV and 2 seconds between each step. Middle: Same type of family in control solution, after applied Cu (2 μM) Phenanthroline (100 μM) in 5 min. Right: Same type of family in control solution after 20 mM DTT is applied in 7 min. c) The suggested paddle model from Jiang et al., 2003a, side view (left), top view (right). In this model the two position 330 and 367 are close to each other (4 – 5 Å), but fail to form a disulfide bond. We are able to get a disulfide bond between 325 and 366 although they are far from each other in this model. For clarity the linker between S3b and S4 is not shown in the top-view figure.
at position 325, had the strongest steric as well as electrostatic effect on the conductance of the Shaker K channel (Figure 8a). This shows that position 325 is electrostatically very close to S4 and that it also sterically affects its movement. The cysteine mutation at position 331 has almost no effect (Figure 8a). This means that position 331 is farther away from S4 compared to position 325.

Since residue 325 showed the biggest effect on S4, we tried to find a residue on S4 with which 325 can form a disulfide bond. One residue we have found so far is 366. Using the x-ray structure from the paddle model from Jiang et al. (2003b) these two positions are relatively far from each other to be able to form a disulfide bond, but still we can see a bond formation (Figure 8b and c). This bond formation is, as in the case of 362C/416C, state dependent and forms only in open state and not in the closed state. Other examined positions, position 330 and 367 does not form a disulfide bond, even though they are close to each other in the paddle model, as seen in Figure 8c.

The paddle model is incompatible with the results from papers I and II. This model predicts that a disulfide bond between S3b and S4 should not alter the opening and closing of a channel, even if the gating itself could be affected. This is because the two helices join each other through the whole movement of the paddle. This means that a disulfide bond would form both in the open and the closed state of the channel and still give raise to an equal amount of ion current in the channel. But the state dependence of the double cysteine mutation 325 and 366 tells that S4 is moving relative to S3b. We can see that the ion current of the channel decreases when Cu/Phenantroline solution is added to the double mutation (Figure 8b). The current reduction should of course not occur according to the paddle model. Furthermore, the paddle is floating freely in the lipid bilayer (Jiang et al., 2003b). In paper I, it is shown that position 362 forms disulfide bond only with the position 416, but not with the neighboring 417. The specific disulfide-bond formation demonstrates a much more rigid movement of the voltage sensor compared to the paddle.

**Electrostatic effects of compounds in the extracellular fluid (Papers III and IV)**

In papers III and IV, I explored how the voltage-sensing mechanism of the Shaker K channel is affected by the positively charged Mg\(^{2+}\) and a negatively charged oxaliplatin metabolite. In paper III, magnesium ions are used, since earlier studies have shown that this ion has a bigger effect on the conductance of the Shaker K channel compared to a lower effect on the conductance of the Kv2.1 (Elinder and Arhem, 2003). The anti-cancer drug oxaliplatin was chosen in paper IV, since it has shown neurotoxic effects which are believed to be caused by surface-charge like properties of the drug (Grollreau et al., 2001; Wilson et al., 2002; Webster et al., 2005; Benoit et al., 2006).

Already in 1957, Frankenhaeuser and Hodgkin showed that metal ions affect channels (Frankenhaeuser and Hodgkin, 1957). Since than it has been shown that various ions affect different channels in different ways (Elinder and Arhem, 2003). Some examples are blockage of the pore, binding to the channel, and screening effect on the S4. Earlier studies has shown
that the two loops closest to the pore of the channel, the loop between S5 and the pore, and the loop between the pore and S6, play the biggest role in the sensitivity of channels to different divalent ions (Elinder and Arhem, 2003). In paper III, comparing the amino-acid sequence of these two loops in the Shaker K channel and Kv2.1, we found three positively charged residues in Kv2.1 which are absent in the Shaker K (see paper III, Figure 1a). By adding three positive charges on the two extracellular loops between S5 and S6 of the Shaker K channel, the usual shift in conductance for the wild type, is in average changed from 13.4 mV to 7.8 mV (Figure 9a). As shown in Figure 9a, the Kv2.1 has also a shift in conductance by about 6 mV. This means that the Shaker K channel is transformed to a Kv2.1 channel, in relation to the sensitivity to Mg ions.

We continued the experiments by investigating the role of each mutated position by itself to see if the charges affect each other. Earlier, it was shown that position 419 is sensitive to extracellular Mg$^{2+}$ (Elinder et al., 2001a). In this study a positive charge at position 419 changed the shift in conductance by about -2 mV. It was also shown that this mutation is independent of the background. This means that 419 is not affected by any of the other mutations. Whether in WT or with other mutation, the change in shift is always about -2 mV for the positively charged 419C (419C-ET+) (Figure 9b). This change in shift agrees well with the results from Elinder et al. (2001a). The other two positions are also independent of the background. 425 shows a change in the shift of conductance by about -4 mV. 451 however gives a very small change, only about -0.1 mV. This means that the position 451 does not have any part in the total change of the Shift in conductance.

While investigating the role of the position 425, we encountered an intriguing question. The different shifts of conductance and changes in the shift is due to interaction of the mutated position with the voltage sensor, but in this case position 425 lays too far away from the voltage sensor to show such a big effect (Elinder et al., 2001a; Long et al., 2005b). Our hypothesis was that this effect must be mediated somehow through other parts of the channel. Indeed, when looking at the electrostatic effect of position 427, the experiments showed that the positive charge of this amino acid is the mediator. A positive charge at position 425 repels the positive charge of the 427 which turns and electrostatically affects the voltage sensor (paper III, Figure 5). A neutral amino acid at position 427 diminished the electrostatic effect of a positively charged residue 425.

In paper IV, I investigated how a platinum-based anticancer drug oxaliplatin, affected the voltage-sensing mechanism of the Shaker K channel. Other studies had reported effects on sodium and potassium channels (Adelsberger et al., 2000; Grolleau et al., 2001; Webster et al., 2005; Benoit et al., 2006). A common finding in these studies is increased ion currents caused by a shift in the channels voltage dependence.

We used oxaliplatin concentrations up to 240 µM on the wild type channel. When no effects could be seen, we studied the monochloro complex, [Pt(ox)Cl]$^-$, of the anticancer drug, since it is a possibility that any effect on the channel could come from this complex. We hypothesized that the monochloro complex could have a surface charge effect on the channel. We also looked at the effect of oxaliplatin and its monochloro complex on cysteine mutations.
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418C and 362C/416C. The reason for choosing cysteine mutations is the fact that sulfur is reactive towards platinum (Mani et al., 2002). In none of the cases could we detect any significant changes in the conductance of the channel. The total lack of effect of oxaliplatin suggests that the reported effects of oxaliplatin are difficult to explain by a common mechanism. Since no surface charge effects are seen, one explanation for the increased ion currents seen by Adelsberger et al. and others mentioned above could be a slowing of the inactivation of the channels and the fact that the effects are relatively channel-specific. In the case of cysteine mutations, the lack of effect suggests that the mutated cysteines are in an unfavorable position to react with oxaliplatin.

Figure 9. a) Left: The shift in the G(V) when adding 20 mM Mg^{2+}. 419C is a cysteine mutation at position 419 in the Shaker K channel showing no change in the shift in conductance compared to WT. 419C-ET^{+}/425K/451K is the triple positively charged mutation in Shaker K channel. ET^{+} denotes a bound MTSET to the cysteine mutation. Right: The shift in G(V) for a single experiment on the wild type Shaker K Channel before (open circles) and after (filled circles) addition of Mg^{2+}. b) The change in charge for each mutation grouped together. The three sign code denotes the charge in each position 419-425-451. 0 = no charge, + = positive charge.
CONCLUSIONS

The results of the Shaker K channel expressed in *Xenopus* oocytes in this thesis show that:

**Paper I**
- Residues in the extracellular end of the voltage sensor S4 (residues 362, 359, and 353) can make disulfide bonds with a residue in the extracellular end of S5 (residue 416).
- The disulfide-bond formation between residue 362 in S4 and residue 416 in S5 is formed in the open state but not in the closed state.
- S4 from one subunit is close to S5 of the neighboring subunit.
- The voltage sensor is not moving during the inactivation of the channel.

**Paper II**
- All amino-acid residues in S3b (residues 323-332) are exposed to the extracellular fluid.
- The amino-acid residues in the C-terminal part of S3b are farther away from S4 than the amino-acid residues in the N-terminal part.
- A residue in the N-terminal part of S3b (residue 325) can make a disulfide bond with a residue in the voltage-sensor S4 (residue 366).
- The 325C-366C disulfide bond is formed in the open state but not in the closed state, suggesting that S4 moves in relation to S3b.
- No disulfide bond can be formed between supposedly close cysteine residues in S3b and S4 as suggested by the X-ray crystal structure of the paddle model.

**Paper III**
- Changing three electro-neutral residues between S5 and S6 on the channel’s extracellular surface (A419, F425, and V451) to positive residues makes the channel Kv2.1 like with respect to its low sensitivity for Mg²⁺.
- The mutated charges do not affect each other.
- A positively charged residue at position 425 affects the voltage sensor electrostatically via its positively charged neighbor residue 427.

**Paper IV**
- Neither oxaliplatin nor its monochloro complex, [Pt(dash)oxCl⁻] affects the voltage-sensor.
- Even though platinum reacts with sulfur, there were no effect on cysteine-mutated channels from either oxaliplatin or [Pt(dash)oxCl⁻].
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