Dynamics and Model of the Pore-Forming Protein Lysenin

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DYNAMICS AND MODEL OF THE PORE-FORMING PROTEIN LYSENIN
DYNAMICS AND MODEL OF THE PORE-FORMING PROTEIN LYSENIN

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microelectronics-Photonics

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ABSTRACT

Membrane transporters are a class of membrane proteins that function to provide a pathway across a cell membrane for the movement of ions and biomolecules. Investigations into the regulatory mechanism of these systems are hindered by their extensive preparation requirements compounded by their fragility and instability. However, lysenin, a pore-forming protein extracted from the earthworm *Eisenia fetida*, provided a unique opportunity to study a protein which is stable in both a soluble and membrane phase. Lysenin channels possess several important properties characteristic of ion channels without the inherent difficulties that plague investigations with biologically vital membrane transporters like voltage-gated ion channels.

Work described here focused on modeling and examining the dynamics of lysenin channels utilizing electrophysiological measurements and theoretical modeling to achieve an understanding of the structure and function of this pore-forming protein. This work investigated the response of the protein channel to an applied electric field which led to current rectification and hysteresis. The results of these studies were used to develop a model describing the mechanisms which give the channel its distinctive functionality. Moreover, the model supported predictions regarding channel behavior that were tested in response to changes in the environmental conditions.

This research uncovered the fascinating behavior exhibited by lysenin channels resultant from the dynamic equilibrium between the channel’s two conductance states. It introduced a model that incorporated the influence of the lipid membrane on the protein channel. The results of these studies validated the model and supported the hypothesized theory as to the origins of channel gating. As a result, this work advanced the general understanding of the fundamental mechanisms of voltage-gated ion channels.
This dissertation is approved for recommendation to the Graduate Council.

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ACKNOWLEDGMENTS

The work detailed in this dissertation is a culmination of my experiences with an intriguing and incredibly complex protein. However, many people have contributed in countless ways to make this work possible.

First, I owe a deep debt of gratitude to Dr. Greg Salamo for his guidance, support and patience. His passion for science has been an inspiration to me. It was a great pleasure to work with Dr. Daniel Fologea, and I would like to express my sincere gratitude for his vast contributions to my understanding and to my development as a researcher. I would like to thank Dr. Ralph Henry for his continuous support and encouragement as well as Dr. David Straub for the many insightful discussions. I am grateful to Radwan Al Faouri for the many conversations, some of which were actually about research. I would like to acknowledge those in the microEP program that have provided a great deal of support over the years. I’d especially like to thank Ken Vickers and Renee Hearon for their tireless efforts to keep me on track. Finally, I would like to thank my parents, Wade and Vera. This achievement would not be possible without their unwavering love and support.

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CHAPTER 1: Introduction

The objective of this research was to explore the dynamics of pore-forming proteins inserted into artificial Bilayer Lipid Membranes (BLMs) by studying their transport properties in response to diverse external stimuli and environmental conditions. More specifically, this work explored the electrophysiological properties of lysenin channels in order to achieve a comprehensive understanding of this unique protein. It furthered understanding into its physiological role, and provided insight into ion channel dynamics. The effects of external stimuli and environmental conditions helped to prepare a model to describe the behavior of voltage-gated pore forming proteins.

1.1 Motivation

The motivation of this work was to advance the understanding of protein membrane transporters. Membrane transporters are an essential component of all living things. They provide a pathway across the cell membrane for the transport of ions and molecules. Membrane proteins constitute approximately 30% of all proteins [1]. However, they represent only about 0.5% of the known and cataloged protein structures [2]. Membrane transport proteins are transmembrane mediators that allow the regulation of ion, nutrient, and waste concentrations within the cell [3]. Ion channels are a category of membrane protein that form a hydrophilic pathway through the hydrophobic domains of the lipid bilayer and control the transport of ions such as potassium, calcium, chloride and sodium across the cell membrane. By regulating ion transport, they are instrumental in creating and maintaining a transmembrane potential which is
essential for healthy cellular activity. In neurons for instance, they play the main role in providing the activation potential used in neural signal propagation [4]. The importance of ion channels has prompted important research focused towards understanding their regulatory mechanism. However, investigations with ion channels, especially single channels, have some serious limitations. Their limited commercial availability provides a substantial barrier to their thorough investigation since it then requires extended preparation for expression and purifications. Additionally, their limited stability in purified form considerably reduces the time-frame available for experimentation. Very often, channel reconstitution in artificial systems requires supplementary tedious steps such as liposome-mediated reconstitution, and the stability in artificial systems is rather poor. To circumvent encountering similar drawbacks during reconstitution in artificial systems, this work focused on studying other transporters able to replicate the fundamental features of ion-channels. It has been reported that lysenin possesses unique characteristics that make them favorable for investigation such as self-insertion and a gating response to stimuli [5-7]. Lysenin shares some of the important characteristics of ion channels, so it provides an opportunity for further understanding specific cellular activity. Additionally, the characterization of lysenin’s properties could prove useful in developing new technologies for sensing, drug delivery and biologically based memory applications [5, 6, 8-12].

Lysenin investigations also provide insight into the mechanisms of protein channel gating. The significance of the gate mechanism to channel functionality can be observed dramatically in ion channel mutations. Mutations in critical ion channel components such as the gate mechanism have been linked to disorders and diseases such as epilepsy, cystic fibrosis, cardiac arrhythmias and some muscle disorders like periodic paralysis and myotonia [13-16]. Mutations interfering with ion channel regulatory function can have ramifications through
increased or decreased voltage sensitivity or in the rate of channel activation or deactivation [13, 15, 16]. Understanding the mechanisms of channel gating will offer new possibilities into the treatment of these diseases [14]. Lysenin channels open opportunities to investigate membrane transporter functionality while overcoming the challenges faced by ion channel research.

1.2 Relevance of ion channel studies to lysenin investigation

Previous work with ion channels has uncovered some fundamental characteristics that were also observed with lysenin. For that reason, the results and outcomes of ion channel studies were used as a model for the lysenin channel investigations. Ion channels exhibit high transport rate of ions and can achieve fluxes on the order of $10^7$ ions/s per channel [4]. The conduction of ions through the channel is regulated by a gating mechanism which responds to an applied voltage, ligand interaction, or the temperature of the local environment [17-22]. Reports focused on this gating mechanism reveal that they contain a mechanical structure that restricts the flow of ions through the channel [23-27].

In vivo, ion channels are regulated by changes in electrolyte concentrations, pH and temperature and can be triggered by molecular interactions [23, 28, 29]. Lysenin’s characteristics were found to be analogous to those of ion channels [5, 6]. Therefore, the investigations with lysenin channels followed a similar course in an effort to expand the understanding of these protein channels. The models and statistical analysis used for ion channels grew into the foundation of the model developed here for lysenin channels. The specific characteristics and behaviors of ion channels should justifiably be elaborated further to provide a foundation for membrane transporter research.
1.3 Ion Channels and Membrane Transporters

Section 1.2 gave a generalized overview of why ion channels studies are pertinent to the investigation of lysenin channels. This section details specific ion channel and membrane transporter functioning as it relates to the lysenin studies conducted in this work.

1.3.1 Voltage-gated ion channels

Voltage-gated ion channels are ion channels which possess a domain capable of responding to applied voltages. The applied voltages initiate a conformational change of the channel which leads to gating to help regulate ion concentration inside a cell. The effects of voltage-gated ion channels were observed by Hodgkin and Huxley from their work with the giant squid axon in 1952. They identified sodium and potassium transmembrane currents from the increase in membrane permeability initiated by the action potential across the neural membrane [30]. This change in the permeability to sodium and potassium ions is the result of transmembrane voltage-gated ion channels that are highly selective toward specific ion species.

Exploring voltage-gated ion channel structure has revealed three basic components: the voltage sensor, the gate mechanism, and the conduction pathway [19]. The voltage sensing domain is part of the channel protein containing an excess of positively charged amino acids [19, 27, 31]. Being charged and coupled to the gate, it responds to an applied electric field and causes the gate to open or close the conduction pathway.

The gate mechanism of ion channels interrupts the ion conduction through the channel pathway. Channel gating is thought to be caused by the obstruction of the channel by a physical mechanism, or by the high resistance of ion flow due to charges within the channel lumen [25, 32]. During the gating response to an electric field, the motion of the voltage sensor on the gate relative to the lipid bilayer produces a measureable current [19, 33, 34]. Physical gates have also
been shown to be exposed to the electrolyte solution because of effects of the solution on the gating current [35]. These domains are the regulatory mechanism for the channel.

The conduction pathway allows the high ionic transport rate through the lipid bilayer membrane. It provides a lower energy barrier for ion conduction than the undisturbed lipid membrane itself [19]. The channel diffusion rate for some voltage-gated ion channels approaches the rate of diffusion in the solution [22]. This is essential for producing the fast response in voltage change required for activities like neural impulses [22].

The structures of some voltage-gated ion channels are now available from diffraction data, but electrophysiological investigations, such as the ones discussed here, were able to produce an approximate overview of ion channels before direct structural investigations were available [26]. The electrophysiology methods and techniques used were based on the BLM technique for the investigation of single channels as an alternative to the patch-clamp technique largely used for studying ion channels on a membrane patch excised from a natural membrane.

Ion channels are, in their simplest representation, a two state system in which the gate transitions the channel between the open and closed states. The probabilities associated with the two states are a result of the measured macroscopic current and have been extensively shown to follow Boltzmann’s statistics [4, 33, 36-38]. The channels, therefore, can be represented as two potential wells separated by an energy barrier where a transmembrane potential changes the well equilibrium relative to each other [33, 36]. The barrier crossing leading to the state transition from the applied voltage leads to some interesting related phenomena initiated by ion channels such as neural and cardiac action potentials and auditory perception [4, 30, 39-42]. In addition to their role in signal transmission, there is even evidence that voltage-gated ion channels help define cellular specialization during development [43-45].
1.3.2 Hysteresis in ion channels

Dynamic hysteresis occurs in a bistable system when the period of an external stimulation exceeds the system equilibrium time [46-48]. Ion channels, being a bistable system, can exhibit hysteresis during the transition between the activated and inactivated states. The two states of the bistable system are thought to be the bottom of two potential wells separated by a barrier as briefly described in the previous section. In ion channels, the rate of barrier crossing can lag behind the rate of change in the external electric field causing hysteresis in the macroscopic current [47]. A plot of the ionic current through a population of ion channels versus applied voltage reveals a hysteresis loop created by the currents corresponding to the increasing and decreasing external voltage. The hysteresis loop area is used as a measure correlation in the lag between the applied voltage and the channel conformation [47, 48]. When the external voltage change is applied much slower than the channel equilibrium time, the hysteresis loop area will approach zero since the channels have been given sufficient time to change conformation. When the voltage changes much faster than the channel equilibrium time, the hysteresis loop area also approaches zero [48]. This is due to the lack of observable voltage gating because the voltage changes too fast for the channels to respond.

1.3.3 Temperature dependent ion channels

The behavior of voltage-gated ion channels has been recurrently demonstrated to abide by Boltzmann statistics [4, 33, 36-38]. As such, ion channels also exhibit some form of thermosensitivity [29]. However, some channels exhibit a greater sensitivity toward temperature than others. The nature of temperature sensitivity in channel gating is for the alteration the relative barrier height thus changing the voltage [29] or ligand sensitivity [49]. Ion channels that demonstrate the greatest temperature sensitivity were found to have activation energies that are
sufficiently different [21]. This provides some indication as to how to interpret experimental results obtained with lysenin channels, and what approaches to take to facilitate further understanding.

The existing model of the gating of thermosensitive ion channels proposes a separate temperature sensitive domain from the voltage sensor [20, 50]. By separating the sensors into two separate but coupled entities, the model can comprise separate activated and inactivated states for each. By coupling the two sensor domains, the voltage and temperature stimuli achieve an additive effect on the energy for the conformational shift [20, 21]. This model was shown to fit existing data, but it is incomplete so additional modifications are needed to correlate with lysenin.

1.3.4 Ligand-gated ion channels

Ion channels whose gating is regulated by a chemical interaction with its sensing domain are considered ligand-gated ion channels. These channels are a critical component for synaptic signal propagation between nerve cells [17, 18]. They are also one of the mechanisms responsible for taste sensation [29, 51]. Lysenin channels also gate in response to a ligand interaction [9, 12]. This provides additional support for using lysenin to analogously study ion channels. However, that work is beyond the scope of this dissertation and will not be discussed further.

1.3.5 Pore-forming protein toxins

Pore-forming toxins, like lysenin, are membrane transporters whose function is detrimental to the host cell. They insert into a cell membrane either to disrupt the electrochemical gradient leading to cell death, or to provide a pathway for a secondary toxin to invade the cell [52]. The self-assembling formation of a channel into a cell membrane is a remarkable process in
itself. These proteins typically are soluble in the aqueous solution surrounding the cell [52-54]. However, in order to insert through a lipid bilayer, the protein must undergo a transformation to allow it to be stable in the hydrophobic region of the membrane. This transformation is not yet well understood, but the resulting transmembrane channel adopts an α-helix or a β-barrel channel structure [52, 54, 55].

The 3-dimentional structure of any transmembrane protein channel can be classified as α-helix or β-barrel which can identify the interaction between the protein and the BLM. The α-helix channel has a helical structure inside the membrane, and the β-barrel is constructed of flat β-sheets rolled into a cylindrical structure. The shapes of the channels provide some insight into the insertion mechanism as it is thought that α-helix channels do not form a membrane bound oligomer before insertion [56]. Lysenin channels share many of the characteristics of pore-forming toxins [54, 56-58] and can offer insights into the structure of the protein by applying current understanding.

1.4 Lysenin

This entire body of work is dedicated to the investigation and characterization of lysenin channels. Lysenin is a 297 amino acid pore-forming protein that is found in the coelomic fluid of the earthworm *Eisenia foetida*. This 33 kDa protein is secreted into the coelomic fluid from chloragocytes and pharyngeal gland cell within the earthworm [59]. Its physiological functionality is not completely known or understood, but it is hypothesized that it plays a role in the immune system of the worm [60]. Transplantation and regeneration experiments during the middle of the 20th century developed an interest into the defense mechanisms of annelids [59].
From these investigations, the protective properties of the coelomic fluid of *E. foetida* were revealed which led to the isolation and purification of the protein, lysenin, in the 1990s [59].

Lysenin exists in its native state as a negatively charged (pI = 5.7-5.8) soluble protein suspended in solution [61]. However, upon interaction with lipid bilayer membrane containing sphingomyelin, it self-inserts to form conductance channels through the membrane. Each channel is comprised of a 6-protein oligomer arranged in a hexagonal pattern giving a channel lumen diameter of approximately 3 nm [62].

As a soluble protein in solution, lysenin must undergo a structural transition to penetrate the hydrophobic domain of the lipid membrane. This transition and insertion process remains largely unknown, but the presence of sphingomyelin is undoubtedly critical for the insertion to take place.

1.4.1 *Sphingomyelin*

A fundamental characteristic of lysenin is the relationship it shares with sphingomyelin. Sphingomyelin is a lipid species found mainly in the outer leaflet of mammalian cell membranes. It comprises approximately 10-15% of the lipids in mammalian cell membranes [63], while it is absent from plant and bacterial membranes. As an integral structural component of biological membranes, sphingomyelin forms localized raft domains which play a role in cell signal transduction [64-66]. Because of the importance role of sphingomyelin in cellular function, lysenin has been used as an investigative tool to determine its presence and to investigate the organization of its lipid membrane domains [67-71].

Even thought the reasons behind lysenin’s targeting of sphingomyelin remain unknown, its precise selectivity towards the lipid is remarkable. In an effort to investigate lysenin’s affinity toward sphingomyelin, several lipids possessing similar structural elements have been
investigated with both planar bilayer membranes and with liposomes, but channel insertion or lysis was not observed [60, 69]. Table 1.1 summarizes the different lipids and their structures studied as a comparison to sphingomyelin. The shaded portions on several of the molecules indicate divergent structural regions from sphingomyelin.

Table 1.1: The molecular structures of sphingomyelin and the similar lipids which have been used to verify lysenin specificity [60, 69, 72, 73]. Shaded regions on a molecule indicate regions of dissimilarity to sphingomyelin.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Molecular Structure</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>Sphingosine</td>
<td><img src="image2.png" alt="Sphingosine" /></td>
</tr>
<tr>
<td>Sphingosine-1-phosphate</td>
<td><img src="image3.png" alt="Sphingosine-1-phosphate" /></td>
</tr>
<tr>
<td>Ceramide</td>
<td><img src="image4.png" alt="Ceramide" /></td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td><img src="image5.png" alt="Phosphatidylcholine" /></td>
</tr>
<tr>
<td>Galactosyl Ceramide</td>
<td><img src="image6.png" alt="Galactosyl Ceramide" /></td>
</tr>
<tr>
<td>Sphingosylphosphorycholine</td>
<td><img src="image7.png" alt="Sphingosylphosphorycholine" /></td>
</tr>
<tr>
<td>Ceramide Phosphorylethanolamine</td>
<td><img src="image8.png" alt="Ceramide Phosphorylethanolamine" /></td>
</tr>
</tbody>
</table>

Several of the structures in Table 1.1 contain identical head groups, while others contain identical sphingosine backbones. It has been demonstrated that lysenin interacts with both the
hydrophilic head of sphingomyelin and the hydrophobic domains [69, 72]. However, interaction does not necessarily indicate channel formation. Lysenin binding occurs exclusively with sphingomyelin, but the membrane fluidity significantly influences the channel formation. Investigations with sphingomyelin containing unsaturated hydrophobic chains revealed lysenin oligomerization is dependent on membrane fluidity [69]. Additionally, cholesterol concentration reportedly influences lipid raft formation resulting in increased localized membrane fluidity promoting lysenin oligomerization [62, 66, 69-71].

1.4.2 Lysenin Channels

Lysenin is considered a pore-forming toxin because of its self-insertion behavior to form an unregulated transmembrane channel which causes cell lysis [7, 60, 61, 63, 67, 68, 73, 74]. However, it also possesses properties of ion channels and membrane transporters such as a high transport rate and voltage and ligand-induced gating [5-7, 9, 12]. Lysenin is stable and active in artificial systems indicating that it does not require other intracellular components to function, and it is commercially available requiring no further purification steps before use. These properties make it an ideal substitute for studying the behavior of natural ion channels.

One of the difficulties in characterizing the behavior of lysenin channels is the lack of structural data of the protein either in solution or as a membrane channel. Lysenin’s primary structure has been identified leading to investigations eliminating its lytic properties or altering its oligomerization behavior [60, 68, 75]. The secondary structure of lysenin was determined to be a combination of β-structures and α-helices [76]. It is hypothesized that the β-structures stabilize the protein-sphingomyelin interaction while the α-helix inserts penetrates the membrane and facilitates pore formation [76].
1.5 Current State-of-the-Art

Lysenin channels have been studied partially due to their strong affinity to sphingomyelin. Their level of study was limited to immunolabeling and fluorescence imaging to detect the presence of sphingomyelin rafts in a lipid membrane. However, more sophisticated imaging techniques have been employed to examine the protein and channel structure in greater detail. Neither technique has yielded a comprehensive image of the lysenin channel and its structure, but each has expanded the fundamental understanding from which this work is based.

1.5.1 Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) has been used to identify the shape and size of the lysenin channels inserted in liposomes with sphingomyelin incorporated in their lipid bilayer [62]. Figure 1.1 is the TEM image showing the lysenin channels distributed in a honeycomb-like arrangement.

![TEM image of lysenin channels](image)

Figure 1.1: TEM image of lysenin channels inserted in a lipid membrane [62]. Scale bar is 10 nm.

Careful examination of the images have concluded that six lysenin proteins assemble into a hexamer shaped channel structure which verify the findings of a six protein oligomer by a SDS-PAGE gel [62].
1.5.2 X-ray Diffraction

Lysenin investigations have recently uncovered the crystal structure of the protein using x-ray diffraction [72]. A cartoon of the protein structure is shown in Figure 1.2 [77].

![Cartoon of the protein structure](image)

**Figure 1.2:** A cartoon representation of the structure of lysenin.

In Figure 1.2, the red flat ribbons represent β-sheet configurations while the violet strands are loops and β-hairpin structures. The blue coiled structures represent $3_{10}$ helix configurations. This study revealed the domains on the protein that interact with the PC head group and uncovered lysenin’s binding with the sphingomyelin hydrophobic tails.

The complexity and intricacy of lysenin channel formation and functionality is an exciting opportunity for investigation. This dissertation explored the relationship between these unique protein channels and external stimuli to gain understanding into their beautifully complex system of movement, memory, and interaction with their environment.
CHAPTER 2: Materials and Methods

2.1 Introduction

This chapter outlines the materials and methods used for lysenin investigations using a planar artificial BLM. It describes each of the components needed to assemble the bilayer chamber and how they relate to each other. The methods and techniques described here serve as guidelines to the experimental procedures performed throughout this research. These approaches evolved over several years to improve the efficiency of lipid bilayer formation while increasing the stability and lifetime of the membrane. Other descriptions of lipid bilayer membranes report difficulty with the stability and longevity of the lipid bilayers [78, 79], but with the methods described in this chapter, those problems were able to be circumvented.

2.2 Equipment and Experimental Setup

2.2.1 Chamber

The experimental chamber was comprised of two identical Teflon fluid reservoirs clamped together separated by a thin Teflon film. These chambers were custom fabricated to specific specifications and designed to have superior functionality to conventional commercial planar bilayer chambers. Being made of Teflon, the chambers were resistant to a wide variety of chemical solvents and provided excellent electrical and thermal insulation. Figure 2.1 shows a scale dimensional diagram of a chamber which was simply a reservoir for the electrolyte solution.
The chamber illustrated in Figure 2.1 was machined out of a solid piece of Teflon. The cylindrical reservoir, outlined with a dashed line in the side view of Figure 2.1, held a total volume of ~1.7 ml. The bottom of the chamber featured a well to confine a Teflon-coated magnetic stir bar which reduced mechanical noise associated with its rotation while allowing the solution to be mixed. A small hole was drilled to position a Ag/AgCl electrode in the electrolyte solution without interfering with the stir bar.

2.2.2 Teflon Film

The lipid membrane was formed over a small aperture that was created in a thin Teflon film. The Teflon film (~120 μm thickness) was fabricated with a single ~70 μm diameter hole approximately centered in its 10 mm x 30 mm area. The hole was fabricated by first inserting a sharp metal needle into the film far enough to make electrical contact with a grounded metal
plate underneath. After retracting the needle, a second needle attached to a high voltage power supply was used to make an electrical arc which creates a uniformly circular hole in the Teflon film. The shape and diameter of the hole was initially verified by optical microscopy until the fabrication protocol yielded consistent results of diameters with less than 5% discrepancy.

2.2.3 Experimental Setup

The Teflon film was used as a barrier to separate two electrolyte reservoirs, allowing only the 70 µm hole as the connection. In preparing the experimental setup, two of the chambers described above were clamped together with a thin Teflon film sandwiched between them to form the complete experimental device as shown in Figure 2.2.

![Figure 2.2: A cross-sectional schematic of the chamber for artificial planar bilayer membranes.](image)

Inside each reservoir an Ag/AgCl electrode was positioned for the application of the voltage stimulus and to monitor the resulting current. Non-polarizable electrodes such as Ag/AgCl were used for several reasons. The electrodes had good stability in the aqueous electrolyte. Also, in order to achieve a uniform mixture in the chamber, the stir bars continually mixed the electrolyte. Non-polarizable electrodes were essential since they did not allow a
stagnant buildup of charge around the electrode. The motion of the ions in solution created a voltage change in the electrode [80]. Appendix H contains a more detailed description of the electrodes.

Before assembly, a thin layer of silicone vacuum grease was used to ensure a seal between the film and the chambers. The chambers were clamped together using an aluminum frame to ensure there was no lateral movement between the chambers during the experiment. Both reservoirs were filled with 1 ml of 130 mM KCl or NaCl electrolyte solution buffered with 20 mM HEPES (shown in Figure 2.2 as the shaded region). Mini stir bars were placed in the wells at the bottom to allow for their free rotation without the risk of making contact with the film and breaking the bilayer. Solution stirring was performed by a Warner Instruments SPIN-2 stirplate designed for low noise applications such as bilayer research. The entire setup was electrically shielded by a Faraday cage which was mounted on a vibration isolation table to reduce electrical as well as mechanical noise. The two Ag/AgCl electrodes were mounted in the electrolyte to both apply the voltage stimulus and monitor the current. One electrode was connected directly to the headstage of a Molecular Devices Axopatch patch clamp system while the other was connected to ground.

Chambers for the experimentation with planar bilayer membranes were commercially available. However, the chambers developed and utilized in this research possessed superior characteristics than a commercial chamber. First and foremost, the aperture used for the formation of the membrane in the commercial chambers is twice the diameter of the apparatus used here. The smaller hole allowed greater stability of the membrane for longer bilayer lifetimes, and was positioned to allow the hole to be easily accessed during bilayer formation.
Overall, our chambers have been designed for ease of use and for ease of cleaning to reduce the chance of cross contamination between experiments.

2.2.4 Axopatch system

The voltage stimulation and current measurements were performed with an Axon Axopatch 200B patch clamp amplifier (Molecular Devices). It was used in voltage-clamp mode with a range of ±200 nA in whole cell β = 0.1 configuration. It was used with a 1 kHz hardware low pass filter. A software low pass filter was used with the cut-off defined to be at least 10 times higher than the sampling frequency. From the patch-clamp amplifier, the current measurements were digitized with a Digidata 1440A digitizer and recorded with the software package pClamp10 (Molecular Devices). The voltage stimuli protocols were established using pClamp10 to define the voltage amplitude, time duration and sampling frequency.

2.2.5 Viscometer

For the viscosity experiments described in Section 6.2, an Anton Paar AMVn (Anton Paar GmbH) automated bench-top viscometer was used to measure the electrolyte viscosity for increasing concentrations of glycerol or glucose. The viscometer measures the time for a rolling ball to travel the fixed length of a glass capillary tube filled with solution. It then uses the solution density and temperature to calculate the viscosity. The viscometer has a measuring range of 0 – 250 s with 0.001 s resolution to calculate viscosities between 0.3 – 2500 mPa·s. The capillary tube was filled with the sample electrolyte solution in accordance with the manufacturer’s operating instructions. The glass capillary tube and steel ball were thoroughly rinsed with distilled water and dried between samples.
2.2.6 Temperature Controller

The effects of changes in temperature on lysenin’s behavior as described in Section 6.3 were performed with a Planar Lipid Bilayer Thermocycler (Warner Instruments). The Thermocycler uses a Peltier element to heat or cool water which is pumped around a jacketed chamber stage. Its manufacturing specifications state it has a temperature range of 5 – 50°C, but in this work, the electrolyte temperature never exceeded 42°C. To minimize evaporation, the chamber reservoirs were covered with parafilm. The solution temperatures were measured with a digital thermometer (VWR).

2.3 BLM Formation

The methods described here for the formation of a lipid bilayer is a hybrid of several other techniques as well as modifications developed over several years of experience. This method allows for the relatively rapid formation of a stable lipid bilayer which will routinely remain intact for over 12 hours [6].

2.3.1 Lipid solution

The BLM is the physical foundation in which the lysenin channels insert. The work performed here formed a planar lipid bilayer over the 70 µm aperture in the Teflon film described in Section 2.2.2. The composition of the lipid bilayer was critical to this work because, as described in Chapter 1, only sphingomyelin is known to be targeted by lysenin.

All of the work described here was performed with a mixture of asolectin, sphingomyelin, and cholesterol. Asolectin is a mixture of soybean phospholipids purchased from Sigma-Aldrich. Its main components are approximately equal concentrations (~25% each) of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI). The
remaining components of asolectin contain small amounts of other phospholipids and polar lipids, however, the exact composition was not identified [81]. Of those lipids, phosphatidylinositol carries a net -1 charge in the pH range used (~7.0) whereas phosphatidylcholine and phosphatidylethanolamine are uncharged. Table 2.1 illustrates the molecular structure of those three main phospholipid components of asolectin.

Table 2.1: The predominant molecular structures of the three main phospholipids comprising asolectin [82].

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Molecular Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td><img src="image1.png" alt="Phosphatidylcholine" /></td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td><img src="image2.png" alt="Phosphatidylethanolamine" /></td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td><img src="image3.png" alt="Phosphatidylinositol" /></td>
</tr>
</tbody>
</table>

Sphingomyelin was incorporated in the lipid mixture for its critical role in the insertion of lysenin. The sphingomyelin used in this work was purchased from Sigma-Aldrich and used without further purification. The details on the structure and hypothesized function of sphingomyelin were given in Section 1.4.1.
In addition to the lipids, cholesterol was included in the mixture for bilayer formation. Cholesterol resides within the bilayer and occupies regions between lipids to help modulate membrane fluidity and provide stability. The cholesterol was purchased from Sigma-Aldrich and used without further purification.

The lipid mixture was prepared by dissolving the asolectin, sphingomyelin and cholesterol as purchased in a ratio of 2:1:1 by mass in n-decane for a total lipid and cholesterol concentration of 25 µg/µl. Other organic solvents were found to evaporate too rapidly which resulted in a solidified lipid crust over the hole in the Teflon film.

2.3.2 Methods

After the chamber was assembled as described in Section 2.2.3, a small amount (< 0.5 µl) of lipid solution was applied directly to hole in the Teflon film on both sides as if painting the lipid solution over the hole. Each of the chamber reservoirs was filled with 1 ml of a 130 mM KCl with 20 mM HEPES buffer electrolyte solution, and the electrodes and stir bars were placed inside. The application of an externally generated triangle wave produced a square-wave current response. The reason for this behavior will be discussed in Section 2.3.3. Using a pipette, approximately half of the solution was drawn out of the reservoir, and slowly injected back in. The level of the electrolyte solution moved up and down exposing and submerging the aperture in the Teflon film. Lipids which were washed into the solution formed a membrane over the hole. The hydrophobic ends of the lipids, which are repelled by the aqueous electrolyte, formed a membrane across the opening by penetrating the hole in the hydrophobic Teflon film.

2.3.3 Multilayer vs. Bilayer

Using the procedure described above, a lipid membrane formed across the aperture in the Teflon film. However, a lipid bilayer was required for the inserted lysenin channel to conduct the
ionic current. Since it was possible for a multilayer to be formed instead of a bilayer, there was a need to be able to differentiate between the two.

The lipid membrane acted as a capacitor in a circuit responding to the applied variable voltage across it. The capacitance of the formed membrane was used to determine its status as a single bilayer or multilayer membrane. The capacitance in the system was observed as the measured current response due to an applied oscillating voltage to the membrane. The oscillating voltage was in the form of a triangular wave with a frequency of 146 Hz and amplitude of \( \sim 72 \) mV. The relationship between the measured current and the membrane capacitance is shown in Equation 2.1.

\[
  i = C \frac{dV}{dt} \tag{2.1}
\]

Here, the current \( (i) \) is directly proportional to the capacitance \( (C) \). The magnitude of the change in voltage with respect to time was constant because a triangular wave was applied (it just switched sign). According to Equation 2.1, the current response to the increasing and decreasing voltage of the triangle wave will be constant thus producing the morphology of a square wave. The observed current response to the triangle wave voltage is shown in Figure 2.3.

A multilayer membrane should have a smaller capacitance than a bilayer because the distance between the outermost layers of lipids is larger, as shown in Figure 2.3a and 2.3b for multilayer and bilayer membranes respectively. The capacitance calculated from Equation 2.1 yielded a membrane capacitance of \( \sim 0.84 \) pF for Figure 2.3a and \( \sim 4.30 \) pF for Figure 2.3b. Here it was possible to determine the configuration of the membrane simply by the observed current response.
Visual observations of bilayer membranes showed the presence of an annulus-shaped mass of lipids around the outer edge of the bilayer along the hole in the film. This thicker region, the Plateau-Gibbs border, contained lipids in a volume and thickness much larger than the bilayer. However, the electrical characteristics observed arose from the thin bilayer portion of the lipid membrane [83].

2.4 Lysenin Insertion

After the bilayer formation, lysenin was then added to the chamber to form channels. As a capacitor, the BLM prohibited the flow of ions between the electrodes. The insertion of lysenin channels into the membrane completed the circuit allowing a macroscopic current to flow.

With the insertion of the protein channels it became important to differentiate between the two sides of the chamber. This was because lysenin was added only to one side to ensure uniformity of the channel orientation. The convention utilized here named the chambers with respect to the headstage. The chamber with the electrode connected to the headstage was designated as the cis side, and conversely the chamber with the electrode connected to ground

Figure 2.3: (a) The current response to the triangle wave voltage for a multilayer. (b) The current response to the triangle wave voltage for a bilayer.
was designated the *trans* side. Figure 2.4 is a schematic of the chamber with an inset of a BLM with inserted channels. The *cis* and *trans* are labeled to visualize the orientation of chambers with respect to the headstage.

![Diagram of the chamber with a schematic of a BLM and inserted channels.](image)

**Figure 2.4**: The chamber shown in Figure 2.2 with an illustration showing a cutaway of a planar lipid bilayer with inserted lysenin channels. Plateau-Gibbs border is not shown for clarity. Illustration is not to scale.

Lysenin was only added to the *trans* side to ensure uniformity within all of the experiments when analyzing results. A -60 mV DC voltage was applied across the bilayer so channel insertion could be observed, and lysenin was added to the *trans* chamber for a final concentration of 0.3 nM. Inserted channels can be seen as a discreet step-wise ionic current shown in Figure 2.5a.
As the lysenin channels were formed in the membrane over time, the concentration of free lysenin in solution decreased as more channels were formed. The current eventually came to equilibrium, shown in Figure 2.5b, indicating the lysenin channel insertions had completed.

2.5 Summary

This chapter outlined the materials and procedures used for the lysenin experiments with planar lipid bilayer membranes. The description of the lipids will prove useful in that some of the behavior of the lysenin channels can be attributed to the interaction between the lipids and the protein. Many of methods described here were modified from conventional methods used for the formation of bilayer membranes [84, 85], but were altered to best suit the equipment and goals of the experiments. Once a stable bilayer was formed, and the lysenin channels had completed inserting, the experiment turned to the investigation of the channel behavior.
CHAPTER 3: Voltage-Induced Gating

3.1 Introduction

Electrical characterization of lysenin has revealed some very interesting and unique properties of this protein. A full understanding of the structure of lysenin is unknown. However, careful examination of the voltage-induced behavior provides a basis for the structural model. This section explores those observed properties in detail, and establishes the boundaries for a theoretical model for the behavior.

3.2 Current Response to Applied Voltage

This work’s first efforts to study lysenin began with a simple I-V curve. The protocol was set to ramp the voltage linearly from -80 mV to +80 mV at a rate of 0.27 mV/s. To ensure all of the channels were initially open, the protocol first ramped the voltage down from 0 mV to -80 mV. The rate of 0.27 mV/s allowed a 160 mV change in the voltage over 10 minutes. This rate gave the channels sufficient time to reach equilibrium as the voltage increased.

The current response to the varying voltage yielded a unique rectification at positive applied voltages. An experimental I-V curve, as shown in Figure 3.1, was the result of the linear voltage ramp as described above. However, a detailed discussion is necessary to fully comprehend the effects the voltage has on the channels, and consequently the current, during the voltage stimulation.

As shown in Figure 3.1, all negative applied voltages (-80 to 0 mV) and small positive voltages (less than ~10 mV) had a linear current response displaying ohmic behavior. This implied that all of the channels were open and contributed to the current. At 0 mV, it was
confirmed that the channels were in the open state because of the observed diffusion of a dye through the channels. Applied voltages greater than 10 mV caused the slope of the I-V curve to change. Between 10 mV and ~19 mV, the slope of the I-V curve decreased indicating that some channels were closing. The current continued to increase, but at an increasingly slower rate for increasing voltages.

![I-V curve](image)

**Figure 3.1:** An I-V curve due to the current response of an applied voltage stimulus at a rate of 0.27 mV/s.

At approximately 19 mV, the current reached a maximum value of ~23 nA. The voltage where the current was maximum (peaked) was labeled as the critical voltage, $V_c$. The critical voltage was the voltage where the increase in current due to the increase in voltage was balanced by the decrease in current due to channel closing. Applied increases in voltage greater than the critical voltage generated a smaller current indicating the I-V curve entered a region of negative conductance. When making a comparison between two I-V curves, the critical voltage was often used as a distinguishing characteristic because of the shift in the negative conductance region to a different voltage.
The response of lysenin channels to a voltage greater than a threshold voltage caused the channels to begin closing (channel inactivation). Applied voltages greater than the critical voltage caused the inactivation of a sufficient number of channels for the net current to decrease despite the continuous voltage increase. In accordance with Ohm's law, an increase in voltage should yield an increase in current. In order for an increase in voltage to result in a decreased current, the dynamic conductance, \( dI/dV \), must take on a negative value. Resultant current for applied voltages over the critical voltage were therefore in the negative conductance region.

The negative conductance region also signified that the lysenin channels were asymmetric in their conductance characteristics. For positive voltages, the channels rectified the current leading to an almost complete eradication of the ionic current. Additionally, it revealed an important insight into the nature of the channel insertion into the bilayer membrane. The asymmetry due to the negative resistance region indicated that the channel orientation was uniform. Lysenin was only added to one side of the membrane, so the insertion of the channels only occurred from one side. Furthermore, the orientations of the channels were all in the same direction.

To further explore the unidirectional insertion, lysenin was added to both sides of the membrane. Figure 3.2 shows the I-V curve from a bilayer membrane where channels were inserted into both sides.

The differences in the currents for the extreme voltages were the result of a non-uniform number of channels on either side. For negative voltages, the channels inserted from the trans side of the membrane were open and conducted, while the channels inserted from the cis side were closed and did not contribute to the current.
For positive voltages, the opposite was true, and the cis inserted channels reopened while the trans-inserted channels closed. The nonlinear regions in the I-V curve were the result of the differences in closing (inactivation) and reopening (reactivation) kinetics. Additional discussion regarding the inactivation and reactivation kinetics will be addressed in Section 3.7.

3.3 Open Probability

The channel gating was described as a bi-stable system consisting of lysenin channels in either the open or closed state. Even on the single channel level, the channels existed as a two state system as demonstrated in Figure 3.3.

Figure 3.3a shows that the individual channel inactivation occurred as a single transitional step as the result of a constant applied voltage of 30 mV.
Figure 3.3: (a) Stepwise decreases in current due to channel inactivation from an applied voltage of 30 mV. (b) Schematic depicting the blockage of the conduction pathway resulting in the observed current shown in (a).

The mechanism of the inactivation, depicted in Figure 3.3b, was the blockage of the channel’s conduction pathway by a voltage sensitive domain in response to an external electric field. The transition between an open and closed state was governed by the rate constants $k_o$ and $k_c$.

$$\frac{k_c}{k_o} \quad \text{Open } \rightleftharpoons \text{ Closed} \quad (3.1)$$

Throughout this work, the open state was the only state that was measurable since only open channels contributed to the current. The total number of channels ($N$) was constant and defined as the sum of the open channels ($N_o$) and the closed channels ($N_c$).

$$N = N_o + N_c = \text{constant} \quad (3.2)$$

The open probability was defined by the ratio of the number of open channels to the total number of channels.

$$P_{\text{open}} = \frac{N_o}{N} = \frac{1}{1 + \frac{N_c}{N_o}} \quad (3.3)$$
Equation 3.1 expressed the transitioning between the open and closed states as an equilibrium reaction occurring at a rate described by the opening and closing rate constants $k_o$ and $k_c$ respectively. Therefore, the reaction rate equation of transition from the open to the closed state was given as the change in the number of open channels with respect to time.

$$\frac{dN_o}{dt} = k_o N_c - k_c N_o$$  \hspace{1cm} (3.4)

At equilibrium, the change in $N_o$ with respect to time along with the change in $N_c$ were both equal to 0, and therefore equal to each other. It follows that the ratio between the number of closed to open channels was equal to the ratio of $k_c$ to $k_o$.

$$\frac{N_c}{N_o} = \frac{k_c}{k_o} = k$$  \hspace{1cm} (3.5)

The ratio of $k_c$ to $k_o$ was defined as the equilibrium constant ($k$). The open probability from Equation 3.3 was defined in terms of the equilibrium constant as:

$$P_{\text{open}} = \frac{1}{1 + k}$$  \hspace{1cm} (3.6)

The kinetic rate constants determined the transition rates over the energy barrier in the bistable system. Figure 3.4 shows a schematic of the energy diagram, where the open and closed states were defined as adjacent energy wells separated by an energy barrier with $\Delta E$ as the difference between the transitional energies.

In Figure 3.4, and in the calculations that follow, $E_c$ was the energy required to transition from the open state to the closed state, and $E_o$ was the energy required to transition from the closed state to the open state.
The height of the barrier and the relative differences in the energies of the states was thought to be dependent on external stimuli such as applied voltage, and temperature. In order to determine those characteristics, the ion current response to an applied voltage was investigated.

From Boltzmann statistics, the probability of finding the channels in the open state (derived in Appendix I.1) can be expressed as:

$$P_{\text{open}} = \frac{e^{-\frac{E_o}{k_B T}}}{e^{-\frac{E_o}{k_B T}} + e^{-\frac{E_c}{k_B T}}} \quad (3.7)$$

Likewise, the probability that the channels are in the closed state is:

$$P_{\text{closed}} = \frac{e^{-\frac{E_c}{k_B T}}}{e^{-\frac{E_o}{k_B T}} + e^{-\frac{E_c}{k_B T}}} \quad (3.8)$$

Where $k_B$ is the Boltzmann constant and $T$ is the temperature.
By combining Equations 3.7 and 3.8, the ratio of the probabilities of finding a channel in the open state to the closed state was associated with the difference in the state energies shown in Figure 3.4 was written as:

\[
\frac{P_c}{P_o} = e^{-\frac{(E_c - E_o)}{k_BT}}
\]  

(3.9)

Where \(P_o\) was the open probability, \(P_c\) was the closed probability, \(k_B\) was Boltzmann’s constant and \(T\) was temperature. Using Equation 3.3, the relationship between the open and closed probabilities and the number of open and closed channels was shown to be equivalent by:

\[
\frac{P_c}{P_o} = \frac{1 - P_o}{P_o} = \frac{N_c}{N_o}
\]

Therefore, from Equations 3.5 and 3.9, the equilibrium constant became:

\[
k = e^{\frac{-\Delta E}{k_BT}}
\]  

(3.10)

Lysenin channels undergo a state transition when an external electric field is applied. This external field transforms the channels by causing a conformational change. The field alters the probabilities by changing the initial difference in state energies by a factor of \(qV\), where \(q\) is the charge of the voltage sensitive domain in the protein and \(V\) is the applied external voltage. The equilibrium constant from Equation 3.10 was then a function of the applied voltage:

\[
k = e^{\frac{-\Delta E + qV}{k_BT}}
\]  

(3.11)

The open probability from Equation 3.6 then became:

\[
P_{open} = \frac{1}{1 + e^{\frac{-\Delta E + qV}{k_BT}}}
\]  

(3.12)
Recall that in Figure 3.1, all of the channels were open for negative and small positive voltages. The ionic current \( I \) passing through the open channels for an applied voltage \( V \) obeyed Ohms law.

\[
I = G V
\]

Where \( G \) was the total channel conductance. Given that only open channels contributed to the current, the conductance was a function of the open probability, therefore Ohms law was written as:

\[
I = V(gNP_{\text{open}})
\]

(3.13)

In Equation 3.13, \( g \) was the conductance of a single channel, and \( N \) was the total number of channels thus providing the conductivity component from the open channels. Combining Equations 3.12 and 3.13 the ionic current corresponding to the state of the channels due to an applied voltage was stated as:

\[
I = VgN\frac{1}{1 + e^{-\frac{-\Delta F + qV}{k_BT}}} + VG_{\text{Leak}}
\]

(3.14)

In Equation 3.14, \( G_{\text{Leak}} \) was the leakage conductance. Due to the nature of disrupting the continuity of the membrane with inserted channels, a measurable leakage current persisted even when all of the channels were in the closed state. The leakage current could also have been due to a non-gating response from a few abnormal channels. To verify that upon complete channel inactivation the slight increase in current was not due to the reactivation of channels at higher voltages, it was observed that the current increased linearly with the voltage following Ohms law. Figure 3.5 shows the theoretical current predicted by Equation 3.14 fit to an experimental I-V curve. The values of \( gN \), and \( G_{\text{Leak}} \) were obtained from analysis of the linear sections of the
experimental data plot for positive voltages. For the theoretical fit $gN = 389.63 \pm 0.37 \, (\mu\Omega)^{-1}$, $G_{\text{Leak}} = 15.76 \pm 0.27 \, (\mu\Omega)^{-1}$, $\Delta E = 0.19 \pm 0.003 \, \text{eV}$ and $q = 10.23 \pm 0.15\,\text{e}$. 

![Figure 3.5: An I-V curve from channel closing data (black) and a theoretical fit from Equation 3.14 (red).](image)

Manipulation of the experimental parameters led to changes in the observable profile of the I-V curve, and the theoretical behavior predicted by Equation 3.14 was analyzed to determine the nature of the channel behavior. The relative energy and the gating charge of the lysenin channels were thought to change due to the effects from external variations. These effects manifested as observable changes in the current response during voltage stimulation. As shown in Figure 3.5, the theoretical representation shown by Equation 3.14 closely corresponded to the experimental data.

Figure 3.6 demonstrated the theoretical changes of the current by altering some of the parameters in Equation 3.14. Figure 3.6a illustrates how the gating shifted for decreasing values of $\Delta E$ with a constant gating charge. Increasing the difference in the open/closed state energy levels shifted the critical voltage towards higher voltages as shown in Figure 3.7a. This implied
that channels possessing increased values of $\Delta E$ required more voltage to transition from the open to the closed state.

![Image of I-V curves](image)

Figure 3.6: (a) Theoretical I-V curves of Equation 3.14 for increasing values of $\Delta E$ for a constant gating charge, $q = 7e$. (b) Theoretical I-V curves from Equation 3.14 for increasing values of $q$ for constant $\Delta E = 0.20$ eV.

Additionally, the negative conductance region became increasingly steeper for higher energy differences. This indicated that the maximum value of the dynamic conductance increased for increasing $\Delta E$. As the difference in the state energies approached zero, the dynamic conductance became smaller, but approached a nonzero value. This signified that gating still took place at a very low positive voltage. Because of this theoretical observation, a potential barrier between the states continued to exist even if there is no difference in the state energies. In the other extreme, if the difference in energies is large, no gating takes place for the range of voltages studied. The model suggests that gating will eventually take place, but in practice, the voltages needed approach those that would break the bilayer membrane.

Figure 3.6b is a plot of Equation 3.14 for different values of gating charges where the difference in state energies remained fixed.
The critical voltage increased for decreasing charge on the gate mechanism shown in Figure 3.7b.

![Figure 3.7](image-url)

**Figure 3.7:** Plots of the critical voltages for changes in (a) $\Delta E$ and (b) gate charge.

The only difference between the curves for changing charge is the voltage at which they gate. This indicated an interaction between the charged gate and the applied electric field. As the gate charge became smaller, a larger magnitude of electric field was required for gating. As shown in Figure 3.6b, the gating for increasing gate charges began at lower voltages. The dynamic conductance region occurred over a larger voltage range for decreased gate charge. However, the maximum (most negative of the negative conductances) dynamic conductance remained constant for each of the gating charges.

The I-V curves from Figure 3.6 imply that the open probability distribution for all negative voltages approaches 1 (all channels open), and decreases toward 0 during the positive voltage application. Figure 3.8 shows the open channel probabilities utilizing the same parameters as in Figure 3.6 for the positive voltage range.

In Figure 3.8a, the open probabilities are shown for a population of channels where the gate charge remains constant ($q = 7e$), but having increasing differences in the state energies, $\Delta E$. 

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The probability curves shown had a uniform profile, but were shifted toward higher voltages for higher values of $\Delta E$.

![Graph showing the probability curves.](image)

**Figure 3.8:** (a) Open probabilities from Equation 3.12 for different values of $\Delta E$ for a constant gating charge, $q = 7e$. (b) Open probabilities from Equation 3.12 for different values of $q$ for $\Delta E = 0.20$ eV.

As the difference between the open and closed energy states increased, the channels were more probable to remain in the open state than for channels with lower $\Delta E$. This indicated that a larger difference in the state energies required more voltage to overcome the potential barrier and switch states. The uniformity of open probability profiles suggested that the mechanism of channel closing remained the same regardless of the energy differences.

Figure 3.8b is the open probability curves for a changing gate charge with a constant difference in energy between the open and closed states, $\Delta E = 0.20$ eV. For a higher gating charge, the open to closed state transition occurred at lower voltages. Hence the critical voltage shifted to higher values for decreasing gate charges. Additionally, that transition had a more narrow range of voltages during which it occurred. For smaller gating charges, the electrostatic interaction of the gate with the applied voltage was much less than for higher charges and yielded a much broader open probability profile.
Using the open probabilities, a new parameter, the midpoint voltage, $V_{\frac{1}{2}}$, was defined as the voltage where the open probability was equal to 0.5, which meant that the opening and closing rates were equal. As shown in Figure 3.9, $V_{\frac{1}{2}}$ was influenced by both the energies (Figure 3.9a) of the open/closed states and the gate charge (Figure 3.9b). The midpoint voltage was a useful parameter for describing the shifted open probability due to stimulus and environmental factors which influenced the lysenin channels observed as changes in state energy and gate charge.

![Figure 3.9: The midpoint voltages for changes in (a) $\Delta E$ and (b) gate charge.](image)

Changes in the I-V curves for a set of experimental parameters were thought to be from changes to the energy states, the gating charge, or a combination of the two. An examination of the changes reflected in the open probability often proved useful in developing a hypothesis as to the origins of the gating mechanism. However, it was difficult to pinpoint the origins of the changes in the gating due to the net effects with $\Delta E$ and $q$ variations. The experiments attempted to isolate the two quantities as much as possible, but the two often remain coupled. During the explanation of the voltage-induced gating, the dynamic conductance was referenced on several
occasions, and the behavior of the current in the negative conductance region was examined more thoroughly.

### 3.4 Channel Equilibration Rates

Recall that in Equation 3.1 the transition between the open and closed states were defined to be governed by the rate constants $k_o$ and $k_c$. Those rate constants were functions of the open and closed state energies. From Equations 3.5 and 3.9, it was inferred that the closing and opening rate constants could be written as:

$$k_c = Ae^{-E_o/k_B T}$$  \hspace{1cm} (3.15)

$$k_o = Ae^{-E_c/k_B T}$$  \hspace{1cm} (3.16)

Where $A$ is a constant. This implied that the opening and closing rate constants were different, and dependent on the energies of activation. It was the difference in the energies that was responsible for the probability distribution observed for the transitioning between states.

In order to examine the inactivation and reactivation rates of the channels, a constant applied positive voltage was used to close the channels. That was followed by a second constant step voltage of low positive or negative voltage to reopen the channels. Figure 3.10a shows an applied +60 mV which caused the channels to transition from the open state to the closed state. The applied voltage protocol is depicted in Figure 3.10b.

The channels then transitioned back to the open state when a constant -60 mV was applied. The applied voltage sweeps described in Section 3.2 were considered as a series of constant instantaneous voltages in sequential increasing or decreasing order. Therefore, the constant voltage applied examined the reaction of the lysenin channels for selected individual voltages included within the sweep range.
Figure 3.10: (a) The current response to the constant applied ±60 mV. A single exponential fit was used to fit the inactivation (red) and reactivation (green). (b) The applied step-voltage protocol.

Initially, when the step voltage was applied, the channels were all in the open state, so the maximum current was initially observed. Over time, the current decayed as the channels closed. The voltage was then switched from +60 to -60 mV, and the reactivation was observed as a reinstatement of the open channel current. Since the inactivation and reactivation was thought to be a single-step process, indicated by Equation 3.1, a single variable exponential fit of the current response to the step voltages was used to obtain the time constant, $\tau$. For channel inactivation, the time constant was $10.7 \pm 0.1$ s and for reactivation the time constant was $\tau = 2.4 \pm 0.3$ s. Channel reactivation was clearly a faster process than inactivation, and was a contributing factor to the origins of the gating hysteresis. The explanation as to why the reactivation was faster than inactivation was related to the energy of the open/closed state transitions (which will be developed in Section 3.7).
A similar experiment was conducted to examine the channel reactivation for small positive voltages. In this case, the inactivation occurred with an applied voltage of +60 mV, then the application of +10 mV opened some of the channels. The current response to the +60 and +10 mV step voltages are shown in Figure 3.11a, and the applied voltage protocol is depicted in Figure 3.11b.

![Figure 3.11](image)

Figure 3.11: (a) The current response to the constant applied +60 mV followed by +10 mV. A single exponential fit was used to fit the inactivation (red) and reactivation (green). (b) The applied step-voltage protocol.

As demonstrated by the figures, the time constants for the channel inactivation were again found to be larger than for reactivation, \( \tau = 10.6 \pm 0.1 \text{ s} \) and \( \tau = 6.1 \pm 0.2 \text{ s} \) respectively. This confirmed that reactivation was a faster process than inactivation. The gating response of the channels to the constant applied voltage shown in Figure 3.10a and Figure 3.11a demonstrated that the channels had a characteristic equilibration time corresponding to the conformational changes of the channel gates. As will be shown in Chapter 5, the difference
between the voltage sweep rate and the characteristic time for each instantaneous voltage was a crucial factor for the dynamic hysteresis.

3.5 Dynamic Equilibrium

In Equation 3.1, the channel state transition was defined to be a dynamic equilibrium. Through the derivation, the conduction state of the channels was governed by a voltage-dependent probability. This suggested that the channels did not undergo a static transition where they remained in that state until they were forced back analogous to a ferroelectric polarization. To investigate the dynamic nature of the channel state transition, the reopening of the channels was examined.

By using a constant applied voltage, the dynamic behavior of the channels was observed as shown in Figure 3.12. The channels in the figure closed and reopened over time in response to a constant 25 mV.

![Figure 3.12: Individual channel inactivation and reactivation from an applied voltage of 25 mV.](image-url)
Even though channel reactivation during a voltage stimulus was observed, that did not explicitly imply a dynamic equilibrium. If the channel reactivation occurred as a result of random thermal fluctuations, it would be expected to be independent of voltage. If however, the reactivation was due to the dynamic nature of the channels, it would be expected to be voltage dependent as the potential well depth shifted as a function of voltage.

Figure 3.13: Histogram of the number of channel reopening events as a function of voltage.

Figure 3.13 is a histogram of the reopening events recorded for individual channels. As expected, the number of reactivation events showed a strong correlation with the applied voltage. This result validated the earlier discussion that the channel state transition was defined by a dynamic equilibrium.

3.6 Time-Dependant Probability

For experiments investigating the equilibration rates, the constant voltages were applied over a time period to allow the channel configurations to come to equilibrium. This indicated that the open probability of the channels was a function of time as well as voltage. The time constants
obtained in Figure 3.10a and Figure 3.11a for channel closing at +60 mV were equal (within the error). For the gating behavior presented thus far, the probability was associated with the applied voltage. Equilibrium current from the constant applied voltage showed that the measured current resulted from an instantaneous applied voltage during the voltage ramp stimulus. Most astonishingly, the time dependent open probability was also a function of the previous state. This section explores the evolution of the open probability and its time-dependant nature. A more detailed derivation of the time-dependent probability and current is provided in Appendix I.2.

Recall that the number of open channels as a function of time was given in Equation 3.4.

\[
\frac{dN_o}{dt} = k_o N_c - k_c N_o
\]

After separating the time component and substitutions for \(N_c\), the change in the number of open channels became:

\[
\frac{(k_c + k_o)}{N_o(k_c + k_o) - N k_o} dN_o = -(k_c + k_o) dt
\]

Integration yielded the time dependant number of open channels as:

\[
N_o(t) = N \frac{k_c}{(k_c + k_o)} e^{-(k_c+k_o)t} + N \frac{k_o}{(k_c + k_o)}
\]

To identify the open probability at equilibrium from the initial open probability a new notation was introduced. The term \(P_{o,(t=\infty)}\) signified the equilibrium probability while \(P_{o,(t=0)}\) signified the initial open probability. The open probability at equilibrium \((P_{o,(t=\infty)})\) from Equation 3.6 related the opening and closing rate constants so it became:

\[
N_o(t) = N(1 - P_{o,(t=\infty)})e^{-(k_c+k_o)t} + NP_{o,(t=\infty)}
\]
In the exponential decay of the number of channels, a new term was defined as the time constant \( \tau \). It related the opening and closing rate constants as:

\[
\tau = \frac{1}{k_c + k_o}
\]  

(3.20)

In the experiments exploring the equilibration rates, the initial applied voltage was 0 mV before the step voltage of +60 mV was applied. This ensured that all of the channels were open prior to the voltage application, and the current measured from the initial step of +60 mV was the instantaneous maximum current of the system. Therefore, the initial open probability corresponded to the previous open probability \( P_{o,(t=0)} \) when the step was applied. In this specific case of the +60 mV step voltage, \( P_{o,(t=0)} = 1 \). In a more general sense, Equation 3.19, was transformed to yield the open probability as a function of time and the probability of the previous conformational state.

\[
P_{\text{open}}(t) = P_{o,(t=\infty)} + (P_{o,(t=0)} - P_{o,(t=\infty)})e^{-t/\tau}
\]  

(3.21)

This demonstrated a remarkable characteristic of lysenin channels. The probability of a channel being in the open state being dependent on the previous state signified that the channels possessed the ability to “remember” that previous state. Examining the equilibrium rate step voltages again, the current was initially at a maximum value. Over time, the current decayed to a minimum value, corresponding to the current at equilibrium \( (t=\infty) \). From this reasoning, the time-dependent current was represented as:

\[
I(t) = I_{\text{min}} + (I_{\text{max}} - I_{\text{min}})e^{-t/\tau}
\]  

(3.22)

Where \( I_{\text{max}} \) was the initial current and thus the maximum value and \( I_{\text{min}} \) was the current after sufficient time for the population of channels to achieve equilibrium. It should be noted that the current was the only measurable quantity throughout the lysenin investigations, so the time
evolution of the current was extremely important in the examination of lysenin channel dynamics.

The rate constants for the two states of lysenin channels were derived by combining Equations 3.6 and 3.20. The opening and closing rate constants became:

\[ k_o = \frac{P_{open}}{\tau} \]

\[ k_c = \frac{1 - P_{open}}{\tau} \]

(3.23)

The rate constants depended on the open probability and the time constant, both of which were voltage dependent.

3.7 Channel Energy Levels

The energy of the open and closed states ultimately determined the gating nature of the channels. Those energies were related to the opening and closing rate constants as given in Equations 3.15 and 3.16. Unfortunately, there was no direct way to measure the open or closed state energy independent of applied voltage. The best method for comprehending the state energies was to calculate the rate constants. Equation 3.23 demonstrates that the opening and closing rate constants could be calculated from the open probability and the time constant. Figure 3.14 shows the calculated rate constants for increasing applied voltages. The error bars shown are within the symbols and were obtained as the standard error from the curve fitting and propagated through the calculations.
As was expected, the opening rate constant was largest at low voltages and was smallest for high voltages. At low voltages, the channels were more probable to reside in the open state, thus channels that closed quickly returned to the open state. Conversely, the closing rate constant was smallest for low voltages and largest for high voltages. During the application of higher voltages, channels that closed were most probable to remain closed. It should also be noted that the rate constants did not reach zero. As an equilibrium reaction, a nonzero probability always remained for a channel transitioning between open and closed states. The plot in Figure 3.14 also shows that the rate constants intersected at ~27 mV. This was the midpoint voltage where the opening rate was equal to the closing rate thus where the open probability equaled to 0.5. The midpoint voltage calculated from the I-V curve of this channel population yielded a value of approximately 27 mV.

Despite the inability to directly calculate the energy levels of the states, it was possible to qualitatively explore the relationship between the open and closed states as a function of increasing applied voltage. As illustrated in Figure 3.14, applied voltages less than \( V_{1/2} \) had a
higher opening rate constant than closing rate constant. This indicated that the barrier height was lower in the closed state compared to the open state as indicated by Figure 3.15a.

![Figure 3.15](image)

Figure 3.15: Schematic diagram of the shift in energy levels due to an applied voltage ($V$). The relative energies of the two states changes for applied voltages less than $V_{1/2}$ (a), equal to $V_{1/2}$ (b), and greater than $V_{1/2}$ (c).

The potential wells had equal energies, shown in Figure 3.15b, when the applied voltage was equal to the midpoint voltage. Increasing the applied voltages to values greater than $V_{1/2}$ resulted in a higher closing rate constant than opening rate constant. This indicated that the barrier height was lower in the open state compared to the closed state as indicated by Figure 3.15c. This reasoning demonstrated that the applied voltage influenced the energies of the channels and caused them to transition into the adjacent energy state.

### 3.8 Dynamic Conductance

In the discussions in the preceding sections, the dynamic conductance was described as the result of the change in the current from a change in voltage. The nature of the channel gating was considered to be the transition of open channels to the closed state from an applied voltage. Since only open channels were considered to conduct current, the conductance of the population of channels was a function of the voltage.
Therefore, the dynamic conductance was defined to be the change in current as a function of the change in voltage from Equation 3.14.

\[
\frac{dI}{dV} = \frac{gN}{1 + e^{-\frac{\Delta E - qV}{k_BT}}} - \frac{gNqV}{k_BT} e^{-\frac{\Delta E - qV}{k_BT}} \left( 1 + e^{-\frac{\Delta E - qV}{k_BT}} \right)^2 + G_{\text{Leak}} \]  

(3.24)

Equation 3.24 is the derivative of the current with respect to voltage. A plot of the dynamic conductance is shown in Figure 3.16, and depicts \( \frac{dI}{dV} \) from the I-V curve fitting in Figure 3.5.

![Figure 3.16: A plot of the dynamic conductance from the curve fit obtained in Figure 3.5.](image)

From the plot in Figure 3.16, the behavior of the channel conductance became apparent. The conductance remained constant for all negative voltages and for small positive voltages reflecting the ohmic nature of the channels. The conductance started to decrease at an accelerating rate until it reached zero, which was defined as the critical voltage in Section 3.2 (the voltage where \( \frac{dI}{dV} = 0 \)). The negative conductance region reached a minimum value at the midpoint voltage. At that point, the dynamic conductance started to increase, but still remained
negative, hence the continuous decreasing current. As the voltage increased, the dynamic conductance approached the value of $G_{\text{Leak}}$ as the channels continued to conduct a small leakage current with constant conductance.

The dynamic conductance was an important aspect in the analysis of the behavior of lysenin channels. It was useful in examining the nature of the gating especially in comparing the response to changes in experimental parameters.

### 3.9 Leakage Current

Equation 3.14 included a term to account for the residual current that existed after all of the channels transitioned into the closed state. The origins of that current were thought to be caused by either unresponsive channels lacking a voltage response or from leakage from the channels or membrane. Single channel experiments showed that the leakage existed even when a single channel was inserted and in the closed state. If the leakage was occurring through the BLM itself due to the disruption of a continuous bilayer by the protein, then it would have occurred during negative voltages as well. However, the magnitude of the current was small compared to the open channel current, so it was not distinguishable. Figure 3.17 shows an I-V curve over positive voltages and indicates the leakage current in red.

From Equation 3.14, the leakage current should follow Ohms law in that it should be linear, and a line extrapolated from the data should pass through the origin as indicated in Figure 3.17 by the dashed red line. However, the current in the region of higher voltage began to increase in a rate much higher than Ohms law would dictate as shown in green in Figure 3.17. This increase in the current suggested an increasingly larger opening through the BLM at higher voltages.
Figure 3.17: I-V curve showing the leakage current predicted in Equation 3.14 in red. Leakage current of unknown origins is shown in green.

At higher voltages, the transmembrane electric field appeared to be causing an increased ionic flux that was suspected to be the result of an increased conduction area. The application of transmembrane electric fields over 250 kV/cm have been shown to cause dielectric breakdown of the lipid bilayer [86]. In the experimental setup described in this work, that electric field corresponded to applied voltages over 125 mV. The current resultant from the higher voltages shown in green in Figure 3.17 was believed to be a reversible semi-breakdown of the BLM from the high transmembrane electric field. To investigate this further, the lysenin channels were exposed to the full range of applied voltage available, ±200 mV. A full range I-V curve is shown in Figure 3.18.
Figure 3.18: An I-V curve using the full range of the Axopatch. The BLM ruptured at ~185 mV.

As shown Figure 3.18, the open channel current was linear for negative voltages between 0 and -35 mV, but became nonlinear when the voltage exceeded approximately -35 mV. For positive applied voltages, the channels underwent inactivation, but the current became nonlinear again at voltages higher than ~75 mV. The nonlinearity of the I-V curve for voltages greater than 75 and -35 mV suggested that the increased current was due ion conduction independent of channel state, but the inserted channels were necessary for the observed conduction. The same BLM with no inserted lysenin channels did not display any conduction for the identical range of voltages applied. This example of the response over the full range required a small number of channels to keep the current within the measurable range. The unevenness of the curve was due to the small number of channels, so individual channel gating was observed. The BLM shown in Figure 3.18 ruptured at a voltage of ~185 mV. The applied voltages were kept relatively low in the lysenin channel investigations to circumvent the effects of high transmembrane electric fields on the membrane. However, the study on neutral lipid membranes required the full range of voltages available to observe voltage-induced gating.
3.10 Neutral Lipid Membranes

As previously stated, asolectin is a mixture made up of several phospholipids. In the pH range used in this work, phosphatidylcholine and phosphatidylethanolamine were neutral and phosphatidylinositol carried a negative charge. To investigate any possible protein-lipid interaction, an uncharged lipid bilayer was used. The neutral lipids were zwitterionic lipids, in that, they carried zero net charge, but did retain locally charged domains on their polar head. The neutral lipid membrane consisted of 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhyPC), sphingomyelin and cholesterol in a 2:1:1 ratio respectively. The bilayer was prepared as discussed in Chapter 2. It was reported that an uncharged bilayer eliminated the voltage-induced channel gating, but did not affect ligand-induced gating [9]. However, that study only investigated gating activity in the ±100 mV range for the purposes of exploring ligand-induced interactions with the lysenin channels. In this investigation, maximum applied voltages of ±200 mV were used to test for gating.

As shown in Figure 3.19, the channels gated at large positive voltages. The response appeared to simply shift the gating behavior to a higher voltage range. The experimental parameters were critical to being able to observe the gating response. The number of channels needed to be low enough that the current from the high voltages remained within the range of the Axopatch (< 200 nA).

Even applying the maximum voltage available, there remained incomplete gating. This implied that even higher voltages would be required to completely close the channels. However, there was a severe risk of breaking the membrane at higher voltages, so further investigations proved to be unfeasible.
The effect of the lipid charge on the voltage-induced gating was hypothesized to influence the localized electric field at the membrane. The lipid charge affected the polarization due to intrinsic bound charges within the membrane. These effects will be discussed in greater detail in the model presented in Chapter 4.

3.11 pH Interactions

All of the experiments discussed thus far were performed at approximately physiological pH (~7.2). The experimental results pointed toward a channel gating domain that was positively charged. This implied that the gating domain on the protein channel consisted of ionized amine groups to provide the net positive charge. Therefore, it was hypothesized that the electrolyte pH should have an influence on any of the protein’s charges exposed to the solution. By analyzing the effects of pH on lysenin gating, further insight was obtained about lysenin’s structure and the origins of its gating. However, changes in pH could also affect charges located on the lipids as well as the protein, so with careful analysis more details regarding the nature of lysenin behavior was uncovered.
To investigate the effect of pH on the channel gating, the electrolyte pH was systematically increased from 7 to 9 after the lysenin had been allowed to insert into the membrane. The resultant gating effects of pH are shown by the I-V curves in Figure 3.20.

![Figure 3.20: I-V curves of lysenin channels corresponding to changes in the pH of the electrolyte solution.](image)

The I-V curves performed at each different pH showed a shift in the channel gating towards higher voltages as shown in Figure 3.20. Leakage currents for the higher pH solutions were not available because of the voltage required for the open probability to reach zero would have broken the membrane. The open channel current measured at -100 mV applied voltages increased 25% over that range of pH as well. This indicated an increase in the channel conductivity which could have been due to a change in the charged gate mechanism, a specific interaction with the lipids, or an increase in the solution conductivity. From the open pore currents measured at negative voltages from Figure 3.20, it was concluded that the macroscopic conductance increased with increasing pH.
Figure 3.21a specifically illustrated that the critical voltage increased for increasing solution pH. Since higher voltages were required for channel gating, this suggests that there was less interaction between the gate mechanism and the applied electric field. The errors in critical voltage are from fitting the I-V curves in Figure 3.20. Errors in the measured pH are smaller than the symbol width.

![Graphs showing critical voltage and gate charge vs. pH](image)

**Figure 3.21:** (a) A plot showing the increase in the critical voltage for increasing solution pH. (b) A plot showing the decrease in the relative gating charge due to increasing pH.

The increased critical voltage with higher solution pH indicated a direct interaction between the voltage sensing domain and the electrolyte solution. To explain the shift in gating, the increased pH was causing a diminished positive charge on the gate requiring a stronger electric field to initiate gating. The values of the gate charge and the errors, shown in Figure 3.21b, were obtained from fitting the I-V curves in Figure 3.20 to Equation 3.14. These results also implied that some charged portion of the gating mechanism was exposed to the solution.

In Figure 3.21b, the gate charge was demonstrated to decrease with increased pH. However, the charge tended to stabilize with higher pH which suggested the presence of an
intrinsically charged domain responsible for the gating. A fit of the charges revealed an inherent charge of about 1.4e.

The pH effects on the channel gating showed that the lysenin was influenced by conditions in the electrolyte solution. It also suggested the gating mechanism contained an intrinsic charge and demonstrated that at least a fraction of it was exposed to the solution. These pH studies provided valuable insight into the nature and origin of lysenin’s gating behavior and structure.

3.12 Summary

This chapter demonstrated the gating nature of lysenin channels in response to an external applied voltage. The origins of the gating were shown to arise from the difference in energies in the open and closed states of the channels, and through the interaction of a charged gate mechanism with the applied voltage. By examining effects from the electrolyte solution used in the experiments, a baseline was established for which to compare further experiments when determining the source of gating.

Lysenin channels were determined to be open at 0 mV and for all negative voltages which pointed towards the physiological role of lysenin in the earthworms. The electric field due to cellular transmembrane voltages is parallel to the direction of lysenin insertion. Thus, the inserted channels would always remain open causing cell lysis and would explain lysenin’s proposed function as an immune defense for those organisms [87].

Voltage-induced gating has provided extremely useful information into the behavior and structure of lysenin channels. The evaluation of the channel response to a varying voltage stimulus made up the foundation of the investigation into lysenin channels. This primary method
led to the investigations of other characteristics of lysenin channels in an effort to comprehend the structure of these unique pore-forming proteins.
CHAPTER 4: Model of Lysenin Behavior

4.1 Introduction

As discussed throughout this dissertation, lysenin channel gating has been a response to an applied electric field oriented anti-parallel to the direction of the channel insertion. It was demonstrated in Chapter 3 that the relative orientation of the electric field with respect to the protein was critical for the reaction of the gating mechanism to the external stimuli. This chapter examines the significant electric fields as they relate to channel gating to generate a viable model explaining the observations. It also investigates the localized electric fields in proximity to the channels and predicts channel behavior due to these fields persisting independent of the electrode potential.

The bistability exhibited by the system was the result of the transition between the two conduction states of the channels. The open and closed states of the channels were described in Section 3.3 as two potential wells separated by an energy barrier whose height governs the transition rates. The electric fields described in this chapter identify the fundamental sources responsible for altering the relative barrier height thus permitting channel conformational changes.

4.2 Membrane Electric Field

The Ag/AgCl electrodes were responsible for applying the primary electric field to the membrane and sensing the resulting current. A discussion pertaining to the interaction of the electrodes with the electrolyte solution is provided in Appendix H. The electric field produced by the electrodes was translated through the electrolyte to localized charged ions external to the
bilayer producing an external electric field ($\vec{E}_{\text{ext}}$). The external electric field was coupled to the applied electrode voltage as $E_{\text{ext}} = \frac{V}{w}$ where $w$ was the width of the bilayer membrane and $V$ was the electrode voltage. Simultaneously, the electric field produced by the electrodes polarized the BLM creating a dipole electric field within the membrane ($\vec{E}_{\text{dipole}}$) [86]. It should be noted that the electrostatic interaction between the ions and the dipoles could not be solely attributed to one or the other. Whether the dipoles were actually induced by the external electric field, or the external electric field was produced by the ion electrostatic attraction to the dipoles was not apparent. Regardless of their origin, the sum of the two fields created an internal electric field which acted on the channels as:

$$\vec{E}_{\text{int}} = \vec{E}_{\text{ext}} + \vec{E}_{\text{dipole}}$$ (4.1)

The orientations of the electric fields are illustrated in Figure 4.1 where the dipole electric field ($\vec{E}_{\text{dipole}}$) is shown in black, the external electric field ($\vec{E}_{\text{ext}}$) is shown in red and the net internal field ($\vec{E}_{\text{int}}$) is depicted in green.

The bound surface charge density ($\sigma_b$) due to the BLM polarization was given as:

$$\sigma_b = \vec{P} \cdot \hat{n} = P_0$$ (4.2)

The polarization ($\vec{P}$) for a dielectric was:

$$\vec{P} = \varepsilon_0 \chi \vec{E}_{\text{int}}$$ (4.3)

Where $\chi$ was the electric susceptibility of the membrane. Combining Equation 4.2 and Equation 4.3, the polarization became:

$$P_0 = \varepsilon_0 \chi E_{\text{int}} = \sigma_b$$ (4.4)
Figure 4.1: An illustration of the electric fields influencing the gating and hysteresis. The relative sizes of the arrows are not to scale.

The electric field due to the dipoles and from the ions external to the membrane was:

\[ \vec{E}_{\text{dipole}} = -\frac{\sigma_b}{\varepsilon_0} \hat{\chi} \]  
(4.5)

\[ \vec{E}_{\text{ext}} = \frac{\sigma_f}{\varepsilon_0} \hat{\chi} \]  
(4.6)

Where \( \sigma_f \) was the free surface charge density due to the ions in the electrolyte. Substituting Equations 4.5 and 4.6 into Equation 4.1, the internal electric field became:

\[ \vec{E}_{\text{int}} = \left( \frac{\sigma_f}{\varepsilon_0} - \frac{\sigma_b}{\varepsilon_0} \right) \hat{\chi} \]  
(4.7)

All of the electric fields were either parallel or anti-parallel to the \( \hat{\chi} \)-direction, so the vector notation was dropped. From Equation 4.4, the internal electric field became:

\[ \frac{\sigma_b}{\varepsilon_0 \chi} = \frac{\sigma_f}{\varepsilon_0} - \frac{\sigma_b}{\varepsilon_0} \]  
(4.8)
Solving Equation 4.8 for the external electric field:

$$\frac{\sigma_r}{\varepsilon_0} = \frac{\sigma_b}{\varepsilon_0 \chi}(1 + \chi)$$

$$E_{ext} = E_{int}(1 + \chi)$$ (4.9)

Equation 4.9 shows that the internal electric field was smaller than the external field because of the dielectric lipid membrane. That is, the external electric field was opposed by the electric field caused by the polarization of the membrane. This signified that the internal electric field was oriented in the same direction as the external electric field.

In Equation 4.9 the susceptibility term, \((1 + \chi)\), was the dielectric constant of the membrane. Experimental and theoretical values of BLM dielectric constants were approximately that of a pure hydrocarbon chain \(\epsilon \sim 2\) [88-90].

### 4.2.1 Electrolyte Resistance and Voltage Drop

Thus far, it had been assumed that the electric field across the BLM was equal to that of the electric field provided by the electrodes. However, the electrolyte solution provided a non-zero resistance that decreased the voltage at the surface of the membrane. Here, the voltage drop due to the electrolyte solution was calculated to verify that the assumption was valid.

The resistance of the electrolyte and the lysenin channels under a DC voltage was represented by the circuit diagram depicted in Figure 4.2. The experimental system consisted of the resistance of the electrolyte on each side of the membrane and the equivalent resistance of the lysenin channels wired in series. Each lysenin channel \((1, 2, 3…N)\) was wired in parallel to each other.

To obtain the solution resistance, the current was measured at an applied voltage while no membrane was formed in the aperture in the Teflon film thus rendering \(R_{\text{channel}} = 0\). The current was measured to be -79.5 nA for a -5 mV applied voltage.
Figure 4.2: The circuit diagram of the BLM system.

From Ohm’s law, the resistance of the electrolyte was calculated to be 31.4 kΩ. Since -60 mV applied across 130 mM KCl, 20 mM HEPES electrolyte solution yielded about -30 pA per channel, a voltage drop of 1 μV/channel at -60 mV occurred across the solution. For 1000 channels, the voltage drop would be 1 mV at -60 mV or a 1.7% change in voltage at the membrane. For 10,000 channels, the voltage drop would be a more significant 10 mV drop, but for that number of channels the current would be out of the measurable range of the Axopatch (±200 nA).

The maximum voltage drop possible for -60 mV that yielded a measurable current would occur with ~6600 channels. With that many channels, the maximum voltage drop possible would be 6.6 mV or 11% of the total applied voltage. However, experiments were typically performed with less than 2000 channels, so the voltage drop due to the electrolyte would be small enough to ignore.

4.2.2 Lysenin Channel Resistance and Heating

Knowing the resistance of the electrolyte solution, the resistance of the lysenin channels could also be calculated. The equivalent resistance of the lysenin channels are in series with the
resistances of the electrolyte. Therefore, the resistance of the lysenin channels was calculated to be approximately 2 GΩ/channel.

The temperature change from the heat transfer due to the current was approximated by determining the maximum power delivered to the channel as:

\[ P = I^2 R \]  
(4.10)

For a single channel, the power transferred was 1.8 pW. Assuming 100% efficiency, the maximum temperature change was approximated as:

\[ \Delta T \frac{kA}{L} = I^2 R \]  
(4.11)

Where \( k \) was the thermal conductivity of a BLM (0.25 W/m·K) [91], \( A \) was the area of a single channel, and \( L \) was the thickness of the membrane. The temperature change due to an applied voltage of -60 mV was \( \Delta T = 0.0051 \) K/channel.

The heating of the channels due to the current did not play a role in influencing channel behavior. Sequential I-V curves did not identify changes in channel behavior indicating that inconsequential quantities of heat were actually being produced or the heat was dissipated to the surrounding electrolyte. It was demonstrated that lysenin channels exhibit thermosensitivity in voltage-induced gating, but only for changes in the equilibrium temperature of the system. Those results will be discussed in detail in Chapter 6.

4.3 Intrinsic Membrane Electric Field

The previous section investigated the electric fields that were present only after an external voltage was applied to the membrane. However, the lipid mixture used most frequently contained PI, whose head possessed a net charge that produced its own electric field within the BLM independent of \( \bar{E}_{ext} \) or \( \bar{E}_{dipole} \). Figure 4.3 is an illustration of a BLM composed of
negatively charged lipids and the resulting electric field inside ($\vec{E}_{lipid}$). Since the lysenin gating occurred at higher voltages with membranes composed of neutral lipids than with charged lipids, the influence of the intrinsic electric field due to the charged membrane was considered.

Figure 4.3: The electric field inside the BLM produced by the intrinsically charged head group.

The two leaflets of the membrane contained a charge in their head groups producing two anti-parallel electric fields from the bound surface charge. However, recall from Chapter 2 that only about 20% of the lipids comprising the BLM had a net charge. Therefore, the surface charge density of the lipids in the membranes used for this work was approximated as two point charges [90] separated by the membrane of width $w$ as shown in Figure 4.4.
Figure 4.4: The charged BLM approximated by two point charges on the head groups of the lipids.

The electric field at any position $x$ within the membrane was the sum of the fields produced by each charge. The electric field from $q_1$ on the left as a function of $x$ was:

$$\vec{E}_1 = -\frac{q_1}{4\pi\varepsilon} \left( \frac{1}{x^2} \right) \hat{x}$$

(4.12)

The electric field from $q_2$ on the right was described similarly as:

$$\vec{E}_2 = \frac{q_2}{4\pi\varepsilon} \left( \frac{1}{(w-x)^2} \right) \hat{x}$$

(4.13)

Where the term $(w-x)$ was the distance of $q_2$ from the position $x$. Therefore, combining Equations 4.12 and 4.13, the total internal electric field produced by the charged lipid head groups was:

$$\vec{E}_{lipid} = \frac{q}{4\pi\varepsilon} \left( \frac{1}{(w-x)^2} - \frac{1}{x^2} \right) \hat{x}$$

(4.14)

Equation 4.14 shows that the electric field due to the charged lipid head groups was a function of position within the membrane. A plot of the electric field as a function of membrane position is shown in Figure 4.5.
The magnitude of the electric field extended to $\pm \infty$ as the position approached the two head groups. In the center, when $x = w/2$, the electric field due to the intrinsic charge on the lipids was expectedly 0.

The addition of the intrinsic electric field in the BLM was hypothesized to cause the gating to occur at lower voltages. This signified that the voltage sensitive domain of the lysenin channel resided in a region of the membrane where $\vec{E}_{lipid}$ was oriented in the same direction as $\vec{E}_{ext}$ which was in the region $w/2 < x < w$.

4.3.1 Voltage-Sensor Position

The observed difference in the gating voltage of neutral and charged lipids was hypothesized to arise from the intrinsic electric field inside of the BLM composed of charged lipids. Since the gating was observed to initiate at lower voltages for charged lipids, the channel’s voltage sensitive domain was thought to be positioned in the region where $\vec{E}_{lipid}$ was oriented in the same direction as $\vec{E}_{ext}$. Using Equation 4.14, the location of the charge sensitive
domain was investigated by analyzing the difference in voltages required to initiate channel gating for neutral and charged lipids.

The voltages at which gating began are shown as the dashed red lines in Figure 4.6. The leakage current that existed in either curve did not play a role in determining the voltage that initiated gating. Subtracting out the leakage changed both voltages by 2 mV, but the difference between the two initiating voltages remained constant.

Figure 4.6: Differences in the gating potentials (red line) between charged lipids (a) and neutral lipids (b).

The voltage that initiated channel gating for the charged lipids was found to be 10 mV, while channels inserted in the neutral lipids did not begin gating until 114 mV was applied. The difference in the applied electric fields when gating began was hypothesized to be the magnitude of \( \vec{E}_{lipid} \), and the position where \( \vec{E}_{lipid} = 0 \) was the location of the voltage sensor. To find the sensor position, Equation 4.14 became:

\[
\frac{q}{4\pi\varepsilon} \left( \frac{1}{(w-x)^2} - \frac{1}{x^2} \right) \frac{\Delta V}{x} = 0
\]  

(4.15)
Where $\Delta V$ was difference in the gating initiation voltages from Figure 4.6. Solving for the position, the voltage sensor was hypothesized to be 0.2 nm from the center of the bilayer as shown in Figure 4.7.

Figure 4.7: A plot of Equation 4.14 showing a hypothesized position of the voltage sensor.

As expected, the calculated position of the voltage sensor was located in the region of the membrane where $\vec{E}_{\text{lipid}}$ was in the same direction as $\vec{E}_{\text{ext}}$. However, the derivation that yielded Equation 4.15 made the assumption that the voltage sensor was positioned in the same plane as the charges on the lipid headgroups. The voltage sensor position shown in Figure 4.7 should consequently be interpreted as the closest possible position to the middle of the BLM.

Lysenin has been shown to interact directly with sphingomyelin [72], which placed the charged lipids further from the voltage sensor, decreasing the $\hat{x}$ component of the intrinsic electric field. Increasing the distance between the voltage sensor and the charged head groups positioned the hypothesized location closer to the membrane surface along the positive $\hat{x}$ direction. In order to pinpoint the exact location of the voltage sensitive domain, more
information would be needed regarding the relative positions of the sphingomyelin raft to the charged lipids.

Investigations with the proteases Proteinase K and thermolysin showed that cleaving the exposed portion of the lysenin protein along the surface of the membrane did not influence channel gating, thus the gating mechanism would be expected to reside within the boundaries of the membrane. Considering these aspects, it predicted that the voltage sensor was located within the 2.3 nm region of the BLM towards the trans side of the chamber.

4.4 Discussion of Voltage-Gated Behavior

The directions of the electric fields were critical to the understanding of the voltage-gating behavior. This was most evident in that the reversal of the direction of the fields induced no gating response from the channels. This was not an unforeseen finding in that lysenin, in its natural environment, would likely experience a transmembrane electric field in the direction that would not generate a voltage-gating response when inserted into a cell membrane [4].

The observed difference in the gating of lysenin channels between neutral lipids and charged lipids was reasoned to be caused by the intrinsic electric field produced by the charged head groups of the charged lipid. By increasing the net electric field inside the membrane, the voltage required to initiate gating would be lower for charged lipids than for neutral lipids, which supported the observed gating behavior.

This model so far characterized lysenin channel behavior due to the application of an external electric field. The behavioral response was due to a sequential increase in the magnitude of the electric field oriented specifically in the direction opposite of the channel insertion. Using this model, decreasing the magnitude of the external electric field was hypothesized not to
simply be a reversal of the observed channel inactivation behavior, but to incorporate an additional mechanism resulting in the hysteresis of the measured current.

4.5 Hysteresis

Experimental results and the corresponding model established that the direction and magnitude of the electric field determined the gating behavior of the channels. However, once the electric field was applied, it was thought that the field persisted locally at the bilayer giving rise to another interesting behavioral characteristic. The channels were observed to reopen at some point, implying that the internal electric field must decrease for the probability distribution to increase. In order to achieve this, either the polarization must decrease or the ions electrostatically bound to the membrane surface must be pulled away. It is reasonable to assume that the membrane polarization does not decrease, so the surface ions must react to an applied force to pull them away.

The membrane polarization and the ions in solution were in a steady-state at the peak of the applied voltage ramp when the channel transitioning had reached equilibrium. At this point, the open probability had reached its minimum for the voltage being applied and the internal electric field had achieved its maximum magnitude. As discussed in Section 4.2.1, the electrolyte resistance was negligible, so the voltage applied at the electrodes was equal to the voltage across the BLM. Figure 4.8 shows a diagram of the BLM in this state.

A small decrease in the applied voltage at the electrodes created a voltage difference in the electrolyte solution. The electrostatic interaction between the fixed charges in the polarized BLM and the free charges in the electrolyte kept a constant voltage across the membrane even though the electrode voltage decreased.
Figure 4.8: An illustration of the ion interaction with the membrane dipoles and the counter-ions at the electrodes.

As shown in Figure 4.9, the difference in the voltage caused an electric field to develop in the electrolyte ($\vec{E}_{dV}$) that opposed the electric field between the fixed and free charges at the membrane/solution interface ($\vec{E}_p$).

Figure 4.9: A schematic of the electric fields acting on the free charges when there is a gradient in electric potential.

Recall that the internal electric field acting on the inserted protein channel was the sum of the electric fields from free charge in the electrolyte and the fixed charge from the polarized lipids. Therefore, $\vec{E}_{int}$ remained constant for small decreases in the electrode voltage.

Further reduction in the electrode voltage increased the magnitude of $\vec{E}_{dV}$ until the field was able to produce a large enough force acting on the free charge to overcome the constant force applied by the polarized lipids as shown in Figure 4.10.
The removal of free charge from the surface of the BLM reduced the internal electric field acting on the lysenin channels.

![Diagram showing electric fields](image)

Figure 4.10: A force on the free charges in solution cause the voltage across the BLM to decrease.

Now the overall voltage across the membrane was beginning to decrease changing the open probability of the channels. Because of the dielectric properties of the BLM, a lag arose between the electrode voltage and the voltage across the membrane. The electrode voltage was applied by the Axopatch and was the voltage where the observed behavior is taking place. Therefore, the charge distribution stored by the interaction between the polarized BLM and the free charge in solution allowed the channels to reopen at lower applied voltages.

The decrease in the membrane’s internal electric field due to the removal of charge was dependent on the decrease in electrode voltage. That implied that the hysteresis would have a static component based only on the value of electrode voltage, not on the rate of decrease. Any dynamic component of hysteresis would be tied to the inactivation rate of the channel for increasing voltages.

4.5.1 Observed I-V Response

During the increasing voltage ramp, the model presented here speculated that during a decreasing voltage ramp the electric field across the membrane due to the ions and polarization was greater than the field supplied by the electrodes. The localized decrease in ion mobility
around the BLM was thought to be a result of the electrostatic force acting on the ions by the polarized lipid membrane.

Towards the completion of the increasing voltage ramp, the channel inactivation was complete and the current increased due to leakage current. The I-V curve shown in Figure 4.11a follows Equation 3.14 and shows that the curve became linear when the leakage current exceeded the channel current due to continuous channel opening and closing.

![Figure 4.11](image)

Figure 4.11: The hypothesized effect of a persistent electric field localized at the BLM on the I-V response during a decreasing voltage ramp. (a) Complete channel inactivation occurred at $V_{\text{inactivation}}$ during an increasing voltage ramp. (b) Decreasing the voltage caused channel reactivation at a lower voltage than inactivation. (c) The I-V curve, consequently took a different return pathway as the voltage decreased.

After the maximum voltage was applied, an I-V curve of the decreasing applied voltage indicated an artificial shift in the channel response since the electric field due to the applied voltage did not reflect the actual electric field across the BLM. Therefore, the channel reactivation initiated at a lower voltage than the complete channel inactivation as shown in Figure 4.11b. The inactivation and reactivation voltages are indicated in Figure 4.11b for comparison. Once the channel reactivation began, the decreasing I-V curve shifted to a lower voltage and therefore had a lower current amplitude than the increasing I-V curve as shown in
Figure 4.11c. Any changes in the environmental conditions were believed to impact the I-V response of the channels. The factors contributing to the channel behavior were considered to be due to changes in the channel gate itself or due to the behavior of the electric fields or both. Changes in the viscosity would be expected to alter the forces shown in Figure 4.8 by introducing additional frictional forces retarding the motion of the ions moving away from the membrane surface and electrodes. This would then translate to a hysteresis curve where the channel reactivation occurred at lower voltages as the viscosity of the electrolyte increased.

4.5.2 Temperature Effects

The discussion of the open probability of the lysenin channels thus far has been confined to constant temperature, but increasing the temperature would reduce the relative barrier height which would allow easier transitions between the states. The statistical model presented in Chapter 3 reflected this reasoning by presenting the open probability is a function of temperature as given in Equation 3.12.

\[ P_{\text{open}} = \frac{1}{1 + e^{-\frac{\Delta E + qV}{k_BT}}} \] (3.12)

Increases in temperature would influence the probability given by Equation 3.12, decreasing the voltage required to close the channels. Conversely, decreasing the temperature would increase the relative barrier height restricting the state transitions. The change in barrier height with temperature corresponded to a change in protein configuration. This meant that \( \Delta E \) in Equation 3.12 was a function of temperature as well and was expected to decrease with increasing temperature. The explicit temperature term in Equation 3.12 reflected a change in the transition rates expressed by Equations 3.15 and 3.16.
The effect of temperature on channel reactivation was hypothesized by considering that reopening was strongly governed by the interaction between free charges in the solution and the fixed charges in the polarized membrane. According to the model, the free charge at the solution/membrane interface gets removed and consequently reduces the internal electric field. In this work, it was assumed that the free charge must be moved a distance $d$ away from the membrane to be released back into the bulk solution as shown in Figure 4.12.

![Diagram of the interaction between the free charge and the force from the voltage difference.](image)

Figure 4.12: The interaction between the free charge and the force from the voltage difference.

The force on the free charge caused by the voltage difference displaced the charge a distance $x_1$:

$$F_{dv} = kx_1 \quad (4.16)$$

The kinetic energy from the thermal vibration of the free charge displaced the charge by $x_2$:

$$KE = \frac{1}{2}kx_2^2 \quad (4.17)$$

Given that the total distance ($d$) to remove the free charge was constant, Figure 4.12 shows that:

$$d = x_1 + x_2 = \text{constant} \quad (4.18)$$

Using Equations 4.16 and 4.17, the distance ($d$) in Equation 4.18 became:

$$d \propto F_{dv} + \sqrt{E} \quad (4.19)$$
The thermal energy of the free charges in the solution increased with temperature as:

\[ E = k_B T \]  \hspace{1cm} (4.20)

The force applied to the free charge was the result of the voltage difference, so

\[ F_{dv} \propto \delta V \]  \hspace{1cm} (4.21)

Therefore, the relationship between the voltage difference and the temperature was:

\[ \delta V \propto C - \sqrt{T} \]  \hspace{1cm} (4.22)

Where \( C \) was a constant. According to Equation 4.22, there should be a constant temperature \((T_{\text{free}})\) at which the free charge in solution has sufficient energy to overcome the coulomb interaction with the lipids \((x_2 = d)\). From Equation 4.22, \(T_{\text{free}}\) was:

\[ C \propto T_{\text{free}} \]  \hspace{1cm} (4.23)

When \( T = T_{\text{free}} \), no force would be required to release the free charge from the membrane \((x_1 = 0)\). A reduction in the electrode voltage would consequently reduce the membrane voltage without a lag between the two as shown in Figure 4.13.

Figure 4.13: When the temperature equals \(T_{\text{free}}\), the electrode voltage and the interface voltage remain equal as the electrode voltage decreases.

As the temperature increased, the lag between the electrode voltage and the membrane voltage decreased and thereby reduced the observed hysteresis. As the temperature approached
$T_{\text{free}}$, the hysteresis was hypothesized to disappear completely. Chapter 6 will discuss the effects of temperature in greater detail.

4.6 Summary

This chapter established the hypotheses that the behavior of lysenin channels was due to its interaction with localized electric fields within the BLM. The applied voltage at the electrodes provided an electric field which, when oriented antiparallel to the direction of insertion, decreased the open probability of the channels leading to an observed decreased ionic current. The interaction between the electric field and the lysenin channel was theorized to work through a voltage-sensitive domain which controlled the conduction state of the channel.

Decreases in electrode voltage, however, were expected to have a delayed effect on the localized electric field from the interaction between the electrolyte ions and the membrane polarization. This model predicted the existence of a static hysteresis for lysenin channels because of the relationship between the decreasing applied voltage and the electric field across the membrane.
CHAPTER 5: Hysteresis

5.1 Introduction

In the previous chapter, lysenin channels were predicted to exhibit hysteresis due to the persistent electric field localized across the membrane after the electrode voltage was decreased. It also described the importance of the electric field direction because of the observed voltage gating presented in Chapter 3. Since it was shown that applied negative voltages did not affect the probability distribution of the channels, this chapter will focus exclusively on applied positive voltages. This chapter on hysteresis will focus on a symmetric applied voltage ramp that increases as well as decreases with time where the net electric field always points in the positive x-direction.

A voltage ramp induced channel gating resulting in the open channel probability distribution as described in Chapter 3. However, changing the direction of the voltage sweep to a decreasing voltage ramp caused the channels to reopen, but along a different probability distribution. This resulted in a hysteresis in the macroscopic current.

Hysteresis is the consequence of a bistable system returning to its original state along a different pathway. For lysenin channels, the reactivation of the channels did not exhibit identical behavior as inactivation. As discussed with ion channels, the dynamic hysteresis was a result of the bistable system undergoing a state transition where the period of an external stimulation exceeded the system equilibrium time [46-48]. Specifically, the increasing voltage induced channel gating when the lysenin channels underwent voltage-induced gating which lagged the voltage changes. Dynamic hysteresis was hysteretic behavior solely due to this delay between the stimuli and the response. Consequently, the gating caused the ionic current to decrease as the
voltage increased. The closed channels underwent reactivation as a result of the reversal in the voltage sweep. However, the ionic current for channel reactivation was lower than for inactivation. This yielded a hysteresis loop where the theoretical area was function of voltage sweep rate [48, 92]. Static hysteresis is a hysteretic behavior that persists even when the sweep rate greatly exceeds the characteristic time of the channels. Previous work with ion channels predicted that the extremes of voltage sweep rates, very slow and very fast, would cause the hysteresis loop area to approach 0 thus indicating only the presence of a dynamic hysteresis [48].

As this chapter will discuss, the rate of channel conformational equilibrium can lag the voltage sweep, thus exhibiting hysteresis by the lysenin channels. Hysteresis is an interesting property that is not unique to membrane transporters [93], but hysteresis in combination with lysenin’s other distinguished properties may provide opportunities to integrate this pore-forming toxin into other applications as will be discussed in Chapter 7.

5.2 Symmetric Voltage Ramp

A voltage ramp from 0 mV to 60mV caused inactivation of the lysenin channels observed as the eventual decrease in the ionic current. A reversal in the voltage ramp to a decreasing stimulus, 60 mV to 0 mV, caused the channels to reopen, but the I-V characteristics from the reinstated ion current differed than that of the increasing voltage ramp. The difference in the measured current from the inactivation to reactivation was hysteresis in the macroscopic current.

Figure 5.1a is a plot demonstrating the stimulus voltage applied to the channels. The voltage sweep protocol was instructed to increase the voltage from 0 mV to 60 mV at a rate of 0.2 mV/s. It then decreased the voltage through the same range at the rate of -0.2 mV/s. The current response to the applied stimulus of Figure 5.1a is shown below it in Figure 5.1b. As
Figure 5.1b illustrates, the increasing voltage (0 - 300 s) caused channel inactivation exactly as was demonstrated for voltage-induced gating.

![Diagram](image.png)

Figure 5.1: (a) The profile of the applied voltage ramp. (b) The lysenin channel response to the applied voltage shown in (a).

The decreasing voltage (300 - 600 s) caused channel reactivation observed as the reinstatement of the current. The description of the mechanism of channel reactivation mirrors that of the inactivation as discussed in Chapter 3. The decreasing voltage caused the population of gated channels to transition back to their open state. The channels’ reactivation was characterized by another negative conductance region followed by a second critical voltage. As the voltage decreased, the channels began to reactivate at a rate faster than the voltage sweep. The channel reactivation reached an equilibrium with the voltage sweep at the second critical voltage. The current returned to a linear profile when the voltage became ~8 mV and all lysenin channels returned to the open state at 0 mV. However, as can be observed from Figure 5.1b, the
ionic current was not symmetric about the maximum voltage, meaning that there were two values of current for each voltage during the sweep.

### 5.3 Hysteresis and Open Probability

The ionic current shown in Figure 5.1b was different depending on whether the voltage sweep was increasing or decreasing. Therefore, it was possible that for any one voltage there were two different induced currents. Being dependent on the voltage sweep direction, one could infer that the induced currents depended on the value of the preceding voltage. Figure 5.2a is a plot of the same data given in Figure 5.1b for examining the relationship between the ionic currents and the applied voltage. Small positive voltages were shown not to influence the channel configuration. As Figure 5.2a demonstrates, voltages less than ~8 mV do not induce gating, therefore, the direction of the applied voltage ramp was not significant for those small voltages and shows that the hysteresis was not the result of permanent structural changes to the inserted channels. However, divergence of the current arose for voltages which induce channel gating.

In Figure 5.2a, the gating began at ~10 mV for the ascending component of the voltage sweep (black line) and continued until ~41 mV, where all of the channels had undergone the state transition from open to closed. The current between 41 mV and 60 mV was associated with the leakage current. The reversal in the direction of the voltage ramp (red line) allowed a continual leakage current from 60 mV to ~35 mV as demonstrated by the ohmic nature of the I-V curve in that range. At 35 mV the channels began to transition from the closed state back to the open state, indicated by the reinstatement of the ionic current.
In the negative conductance region, the rate of channel reactivation exceeded the voltage sweep rate similar to the channel inactivation. Finally, at ~5 mV all of the channels had returned to the open state and the ohmic behavior returned.

Figure 5.2: (a) The I-V curves corresponding to an ascending (black) and descending (red) voltage ramp showing the channels' hysteresis. (b) The open probability for an ascending (black) and a descending (red) voltage ramp as a function of voltage from Equation 3.12.

The open probabilities of the channels are shown in Figure 5.2b. Despite the fact that the channel behavior arose from one complete cycle of voltage stimulus, the inactivation (black) and reactivation (red) I-V response and probabilities were analyzed independently of each other to identify differences in the curves. The open probabilities calculated from Equation 3.12 suggested that \( \Delta E \) changes between inactivation and reactivation. However, there was no reason to believe that the direction of the voltage sweep influenced the difference in the open/closed state energies. To account for this inconsistency, the origins of the hysteresis were explored further since something within the system was changing to make reactivation different than inactivation.
The applied positive voltage from the electrodes was translated through the electrolyte to produce a region of charge at the surface of the membrane. This produced an external electric field across the membrane. The BLM also became polarized causing an electric field due to the dipole of the lipids. The interaction of these electric fields caused the localized electric field near the channels to remain larger than the electric field produced by the electrodes. It was only when the voltage was decreased enough that the ionic charges on the bilayer began to dissipate.

A localized voltage caused by the ionic electric field was stated to lag the applied voltage by $\delta V$. Therefore, the open probability in Equation 3.12 for channel reactivation was expressed as:

$$P_{open} = \frac{1}{1 + e^{-\frac{-\Delta E + q(V + \delta V)}{k_B T}}}$$

(5.1)

The measured ionic current given by Equation 3.14 then became:

$$I = V gN \frac{1}{1 + e^{-\frac{-\Delta E + q(V + \delta V)}{k_B T}}} + V G_{Leak}$$

(5.2)

The analysis of hysteresis data was then performed using Equation 3.14 for inactivation and Equation 5.2 for reactivation. The inactivation and reactivation data in Figure 5.2a fit with Equations 3.14 and 5.2 respectively are shown in Figure 5.3.

Forcing a best-fit to prior data in Figure 5.3a yielded values of $\Delta E = 0.1977 \pm 0.0004$ eV, $q = 8.13 \pm 0.02$ e, and the fitting in Figure 5.3b yielded $\delta V = 5.380 \pm 0.005$ mV. The reported error was the standard error given from the curve fitting in Origin.
Figure 5.3: (a) The inactivation curve in Figure 2a (black) with the theoretical fit (green) from Equation 3.14. (b) The reactivation curve in Figure 2a (red) with the theoretical fit (yellow) from Equation 5.2.

This demonstrated that the open probability distributions reflected identical channel behavior but the reactivation was shifted by 5.38 mV. Upon closer examination of the probabilities in Figure 5.2b, it was evident that the two probability distributions are separated by a value of 5.38 mV at their midpoint voltages shown in Figure 5.4.

Figure 5.4: The open probability from Figure 5.2b showing the 5.38 mV voltage separation.
5.4 Dynamic Hysteresis

In Chapter 3, it was shown that the open probability was time dependent, so the rate of the channel closing affected the ionic current with respect to the voltage ramp rate. When the rate of the increasing voltage ramp exceeded the channel closing rate an increase in current was observed. The decrease in current due to the negative conductance region was shown to be due to the channel transformation rate exceeding the voltage increase rate. To examine how the rate of the voltage ramp affected the hysteresis, modifications were made to the voltage sweep rates. The voltage ramp was always kept symmetric with respect to the increasing and decreasing sweep rate in order to be able to make a comparison between inactivation rates and reactivation rates. Figure 5.5 shows the resulting hysteresis from sequentially decreasing voltage ramp rates. In all of the panels in Figure 5.5, black lines denote the increasing voltage segment and red denotes the decreasing voltage segment. Figure 5.5a has corresponding arrows to symbolize the direction of the voltage sweep as well.

Figure 5.5a shows that there is no hysteresis for the fastest rate of 1000 mV/s. The current response to the increasing and decreasing voltage ramps was symmetric. This indicated that the rate of voltage change exceeded the equilibration time of the channel. The ohmic behavior was the result of the voltage changing so quickly that the ramping cycle was complete before the channels were able to respond. By slowing the voltage rate by a factor of one hundred to 10 mV/s, a definite hysteresis occurred as shown by Figure 5.5b. The slower voltage sweep allowed the gating to influence the current and caused the hysteretic behavior shown here. From gating, the maximum current decreased and the current response was no longer symmetric. However, the decreasing voltage ramp showed that the channels continued to close during the higher
applied voltages. This indicated that the rate of voltage change remained faster than the equilibrium time of the channels but slow enough for observable hysteresis.

Figure 5.5: Hysteresis of lysenin channels for varying voltage rates. (a) 1000 mV/s. (b) 10 mV/s. (c) 0.2 mV/s. (d) 0.05 mV/s. Increasing voltages are indicated as black, and decreasing ramps are indicated as red as the arrows signify in (a).

Decreasing the voltage rate to 0.2 mV/s, as shown in Figure 5.5c, changed the hysteresis profile of the current. The slower increasing voltage ramp completely inactivated the channels at ~36 mV, and the reactivation began during the decreasing voltage segment at ~29 mV. The voltages greater than 36 mV induced a leakage current which became increasingly noisy at higher voltages. As with Figure 5.5b, the sweep rate still exceeded the equilibrium time of the channels. That became more apparent when the rate was decreased to 0.05 mV/s, as shown in
Figure 5.5d. Here the voltage of complete inactivation was slightly shifted toward lower voltages along with the start of channel reactivation (~33 mV and ~27 mV respectively). As with the previous sweep rate, once the channels were closed the applied voltage induced a leakage current. When the channel characteristic equilibrium time exceeded the rate of the voltage stimulus ramp, the hysteresis curves remained constant.

5.5 Static Hysteresis

Decreasing the voltage sweep rate was demonstrated to allow the channels to achieve a conformational equilibrium during each instantaneous applied voltage. However, lysenin differed from ion channels as the hysteresis demonstrated by ion channels disappeared for slow voltage sweeps [48]. To verify this finding, a very slow voltage sweep was applied to the lysenin channels over ~12 hours. Figure 5.6a shows the hysteresis for a rate of voltage change of 3.8 µV/s. Even though the voltage rate was well below the equilibration time of the channels, the hysteresis persisted, indicating that lysenin channels had an intrinsic static hysteresis.

Observations of the hysteresis were best compared to each other by using the area of the hysteresis loop similar to the analysis of ion channel hysteresis [92, 94]. When the voltage sweep rate was slower than the characteristic time, the hysteresis loop areas were equal which signified that lysenin channels had an intrinsic static hysteresis.

It has been reported for ion channels that a plot of the loop areas should yield a “bell-shaped” profile [48]. As shown in Figure 5.6b, lysenin channel behavior yielded a “bell-shaped” profile, but differed from ion channel results by the evidence of a persistent static hysteresis. For the highest sweep rate, the area was expected to be zero due to the rapidness of the voltage
sweep with respect to the channel equilibration time. Without allowing for conformation changes at each instantaneous voltage, no hysteretic behavior was observed.

![Graph a: Hysteresis for 3.8 µV/s voltage ramp. Graph b: Plot of the hysteresis loop area as a function of voltage sweep rate.](image)

Figure 5.6: (a) Hysteresis for 3.8 µV/s voltage ramp. (b) Plot of the hysteresis loop area as a function of voltage sweep rate.

As illustrated in Figure 5.6b, the loop area increased to a maximum as the voltage sweep rates decreased. The hysteresis loops with large areas were with sweep rates where channel inactivation was continuing during the decreasing voltage sweep segment, thereby having two elements contributing to the decrease in current. Continuing to decrease the sweep rates, the loop areas responded by getting smaller. When the sweep rate was on the order of the equilibration time, the increasing voltage ramp segment caused complete inactivation, but it was continuous over the time-course of the increasing ramp. Voltage sweep rates less than ~0.1 mV/s yielded equal hysteresis loop areas, demonstrating that hysteresis persisted even at low voltage sweeps. This provided convincing evidence that lysenin channels possessed an innate static hysteresis which was not observed with ion channels. Due to the nature of the lysenin experiments, the minimum frequency of the voltage sweep was governed by the stable lifetime of the bilayer lipid membrane. Hysteresis may disappear during a longer voltage sweep, but it was not observed in
the system described here. With lysenin channels, the persistent hysteresis at long sweeps signified that the hysteresis was an intrinsic part of the channels, and indicated that the state of the lysenin channel system was always dependent on the channel history.

5.6 Single Channel Hysteresis

As previously stated, hysteresis was hypothesized to be a consequence of the lysenin channels having two probability distributions, one for increasing voltages and one for decreasing. In order to determine if the hysteresis was an intrinsic characteristic of the channels or rather simply the net result of the difference in reaction rates, the behavior of a single channel was investigated as shown in Figure 5.7.

![Figure 5.7](image.png)

Figure 5.7: (a) An I-V curve from increasing (black) and decreasing (red) voltage for a single lysenin channel. (b) The open probability distributions corresponding to increasing (black) and decreasing (red) voltage ramps.

Single channel investigations were performed exactly as multichannel experiments. Because the ionic current through a single channel was small (~30 pA/channel), signal noise was much more apparent in the measurements. By observing the current, the instantaneous open
probability was determined directly from the I-V curves for ascending and descending voltages. Figure 5.7a shows the current response from an ascending (black) and descending (red) voltage sweep.

Sequential voltage sweeps may yield different experimental results where the channel inactivation and reactivation occur at different voltages. An instantaneous open probability was assigned to each value of applied voltage corresponding to the observed state of the channel (open = 1 or closed = 0). The probability distribution was calculated by averaging the instantaneous probabilities at each voltage from 140 sweeps. Figure 5.7b shows that a single channel exhibited two probability distributions dependent on the direction of the voltage ramp. The error bars shown are the standard deviation from the mean of the instantaneous probabilities. This signified that the observed hysteresis from a lysenin channel population originated from the combined open probabilities of each single channel.

5.7 Summary

The reversal of voltage ramp stimulus that caused channel gating caused lysenin channels to exhibit hysteretic behavior. This behavior was thought to originate simply from the response lag between the channel gate and the applied voltage stimulus, but was observed to be an intrinsic characteristic of lysenin channels. The change in the open probability distributions suggested that the membrane was playing a role in the local electric field close to the channels. This was demonstrated by treating channel inactivation and reactivation as two probability distributions separated by a residual electric field. Building upon the theoretical background presented in the previous chapter, the time dependant open probability was found to be dependent on the probability of the previous step. This chapter has provided evidence that the
state of the channel was influenced by the previous state, in that hysteresis was observed as a fundamental behavior of individual channels.

The hysteretic behavior indicated that lysenin channels exhibited memory, where their current state was influenced by previous stimuli. The notion of molecular memory coupled with the intrinsic static hysteresis opens possibilities for the incorporation of this distinctive pore-forming toxin into novel and exciting biological-based technologies.
CHAPTER 6: Environmental Factors

6.1 Introduction

The previous chapters established the foundation for the electrical characterization of lysenin channels. The voltage-induced gating was shown to be the result of the channels response to an external electric field. Those electric fields, in turn, persisted in the vicinity of the channels due to the polarization of the membrane thus producing hysteresis in the ionic current. The insight into the behavior of the gating mechanism evolved from investigating the changes in current due to different applied voltage profiles. However, a more specific and targeted approach is required to gain a full understanding of the underlying mechanics of lysenin channel gating. This chapter will explore the effects of external factors in addition to the applied voltage on lysenin channels in order to get a more complete picture of the gating. It will introduce the effects of solution viscosity to determine the presence of a physical gating mechanism, and demonstrate the relationship of temperature to the open/closed state transition.

6.2 Viscosity

Without detailed information as to the structure of a lysenin channel, it is difficult to identify the presence of a physical structure responsible for the channel gating, and has been purely hypothesized to this point. Previous work with ion channels revealed that gated channels possess a charged structural domain that occludes the channel to prevent ionic flow. The observed similarities of lysenin to the reported ion channels led to the speculation that such a structure exists in the channels described here. To identify a possible structural element in lysenin, it was hypothesized that the motion of a mechanical gate could be hindered by changes
in the environmental conditions. The voltage-induced gating of a physical structure should be impeded in the presence of a viscous fluid. The existence of a physical channel gate would yield an increased time constant for inactivation and reactivation as the viscosity of the fluid increased. The very nature of lysenin channels required that the investigations take place in solution, but any effects as the result of the fluid viscosity would not have been observed since all of the solutions had comparable viscosities. Therefore, increased viscosity studies were analyzed relative to a control electrolyte.

6.2.1 Conductivity Effects

The conductivity of the electrolyte changes with the viscosity. The Stokes-Einstein relation between the viscosity and the diffusion coefficient \((D)\) is given as [95]:

\[
D = \frac{k_B T}{6\pi r \eta}
\]  
(6.1)

Where \(T\) is the temperature, \(r\) is the ion radius and \(\eta\) is the viscosity.

The electrical conductivity of the electrolyte \((\Lambda)\) can be written in terms of the cation and anion diffusion coefficients \((D_+ \text{ and } D_- \text{ respectively})\) as the Nernst-Einstein equation given as:

\[
\Lambda = \frac{ze_0 F}{k_B T} (D_+ + D_-)
\]  
(6.2)

Here \(z\) is the charge number of the ions, \(e_0\) is the electron charge, \(F\) is the Faraday constant, \(D_+\) is the diffusion coefficient of the positive ions and \(D_-\) is the diffusion coefficient of the negative ions. Combining Equations 6.1 and 6.2, the conductivity as a function of viscosity was:

\[
\Lambda = \frac{ze_0 F}{6\pi} \left( \frac{1}{R_+} + \frac{1}{R_-} \right) \frac{1}{\eta}
\]  
(6.3)

In a 1:1 salt such as KCl, the cation and the anion both contribute to the conductivity [95]. Therefore in Equation 6.3, \(R_+\) was the ionic radius of the cation and \(R_-\) was the ionic radius.
of the anion. The electrolyte solutions used in this work were exclusively KCl or NaCl. The ionic radii of the ions composing those salts are shown in Table 6.1 below.

Table 6.1: Ionic radii of select ions [96]

<table>
<thead>
<tr>
<th>Ion</th>
<th>Radius (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K⁺</td>
<td>1.33</td>
</tr>
<tr>
<td>Na⁺</td>
<td>0.95</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>1.81</td>
</tr>
</tbody>
</table>

In Equation 6.3 the electrolyte conductivity was inversely proportional to the solution viscosity. This was verified experimentally where the viscosity of a 130 mM KCl, 20 mM HEPES electrolyte solution was increased from 0.97 to 1.65 mPa·s by the addition of glucose. Since glucose did not contribute ions to the electrolyte, increasing concentrations of glucose only altered the viscosity. The measured conductances are plotted in Figure 6.1.

![Figure 6.1: Plot of the electrolyte conductivity as a function of inverse viscosity.](image-url)
As expected, the plot of solution conductivity versus the inverse viscosity was linear as predicted by Equation 6.3. A linear fit produced a slope of $14.6 \pm 0.1 \text{ C}^2/\text{m}^4$. To verify that the addition of glucose only increased the viscosity, the conductance of the channels was determined from the slope of the linear portions of the I-V curves and plotted against the corresponding inverse viscosity, shown in Figure 6.2.

![Graph](image)

Figure 6.2: The channel conductance as a function of inverse viscosity.

The linear relationship between the channel conductance and the inverse viscosity signified that changes in channel conductance were resultant from the increased viscosity by the addition of glucose. Any effects observed in the channel gating could only be attributed to the increased viscosity of the electrolyte solution.

### 6.2.2 Viscosity Experiment

In an effort to increase the solution viscosity it was first important to design the experiment as not to change the energetics of the lysenin channels or influence the gating by a ligand interaction. Changes in viscosity were easily achieved by changing the solution
temperature, but it would also have affected the gating due to the resultant change in energy as will be shown in Section 6.3. The addition of a more viscous fluid to the electrolyte would controllably increase the solution viscosity, but the composition of the added fluid must not increase the electrolyte concentration nor cause gating on its own as observed with multivalent ions. [12]. Therefore, increasing the viscosity of the electrolyte was accomplished with electrolyte solutions containing either glycerol or glucose. Higher concentrations of glycerol tended to shift the gating voltages outside of the working range for a lipid bilayer. Additionally, the addition of glycerol consistently yielded data with a much larger noise suggesting a secondary interaction with one of the components of the system. This work focused on the addition of glucose as the method of increasing solution viscosity, but equivalent overall effects were observed for low concentrations of glycerol.

The viscosities of the solution with increasing concentrations were measured with an Anton Paar AMVn viscometer at 22°C. The average measured viscosities are displayed in Figure 6.3 as a function of viscogen concentration, and the error bars are the standard deviation from the average.
Figure 6.3: The measured viscosities for solutions of increasing glucose concentration in 130mM KCl, 20 mM HEPES at 22°C.

The viscosity data were fit to a polynomial correlation curve to calculate the viscosities used in the experiment. The correlation curve was only used for the calculation of the solution viscosities used for the investigations. As expected, the viscosity of the solution increased as the glucose concentrations increased.

6.2.3 Results

The lysenin channel experiments for increased viscosity were performed using identical electrophysiology configurations as previously discussed in Section 3.4 for I-V curves and time constant measurements. The electrolyte viscosity was successively increased after the lysenin channels had inserted into the membrane. I-V curves for increasing viscosities of the electrolyte solution were recorded with an applied voltage sweep rate of 0.2 mV/s, and are shown in Figure 6.4. The decreases in the open channel current were due to the decreased mobility of the charge carriers from the increased viscosity.
Figure 6.4: I-V curves for increasing electrolyte viscosity.

The I-V curves in Figure 6.4 showed a shift in the critical voltage toward higher voltages for increasing solution viscosities. A plot of the critical voltages as a function of viscosity is shown in Figure 6.5. The error bars represent errors in the critical voltage from fitting the I-V curves in Figure 6.4 and the errors in viscosity are from the correlation curve.

Figure 6.5: The critical voltage increases as the viscosity increases.

Between 0 mM and 182 mM glucose concentration, the viscosity increased 7% from 0.97 to 1.04 mPa·s while the conductivity at low positive voltages decreased by 4% as shown in
Figure 6.4 (black and red lines). However, the increased in viscosity yielded a 13% larger critical voltage indicating that the gate mechanism was taking longer to close.

Figure 6.5 suggests that the increased viscosity required higher voltages to induce channel closing. However, the increased critical voltages were an artifact due to the rate of the voltage sweep. The increased viscosity increased the equilibration time of the channels, consequently requiring a slower voltage sweep to yield undistorted observations of the effects on the channels. Therefore, I-V curves shown in Figure 6.4 did not accurately illustrate the response of the channels to the applied voltage. To examine this closely, the increased viscosity was held constant while the rate of the voltage sweeps was changed. Figure 6.6 shows how the critical voltages changed for decreasing rates of voltage sweeps with a solution viscosity of 1.10 mPa·s (347 mM glucose).

Analogous to the hysteresis curves in the previous chapter, the rate of the voltage sweep effected the observable channel gating. A decrease in the sweep rate from 0.2 mV/s to 0.033mV/s shifted the observed critical voltage toward lower voltages, and complete channel inactivation occurred over the range of voltages studied. A plot of the critical voltages was expected to decrease as the rate decreased and become constant for rates slower than a threshold value.
Figure 6.6: The critical voltage for decreasing sweep rates at a constant viscosity of 1.10 mPa·s.

To thoroughly examine the lag between the voltage sweep rate and the conformational equilibrium time, the inactivation time constant was determined. Similar to the protocol described in Chapter 3, a +60 mV step voltage was used to examine the closing rates of the channel for increasing solution viscosities.

Figure 6.7a shows the closing kinetics of the lysenin channels as the result of a constant +60 mV applied voltage. The increased viscosity caused a decrease in the closing rate which can be observed by the expanded scale shown in Figure 6.7b. Despite the changes in channel closing rates, the applied voltage caused the channels to undergo complete inactivation. The kinetics curves were fit to a single exponential function to determine time constant. Figure 6.8 shows the average time constants as a function of viscosity from the addition of glucose. The error bars are the standard deviation in the time constants obtained from curve fitting.
Figure 6.7: (a) The current response due to a +60 mV constant applied voltage. (b) An expanded view of the kinetics curves shown in (a).

As Figure 6.8 shows, the time constant increased with increasing viscosity. The absence of ligand induced gating by the addition of the viscous medium, along with the reversibility of the critical voltage shift, indicated a need to account for the mechanical motion in the time dependent probability. The fluidity of the solution played a substantial role in the characteristic time of the channels, but did not influence the equilibrium probability.
The voltage-induced gating was not affected by the solution viscosity, and the resultant current was only delayed in time. The equilibrium probability due to solutions of different viscosities was shown to be equal.

Lysenin’s gating sensitivity to increasing viscosity indicated that there is a physical gating mechanism responsible for blocking the channel. In an attempt to determine the location of the gate relative to the channel, the local viscosity of the solution was changed. Utilizing a viscogen composed of a large molecule has been shown to not affect the gating of ion channels [35]. As large molecules could not enter the lumen of the channel, no change in gating response indicated the location of the gate must be inside. Due to the relatively large diameter of lysenin channels, determining a suitable uncharged viscogen was unsuccessful.

Ion channel gating mechanisms often have a structural component which protrudes into the bilayer. Similar to changing the solution viscosity, the fluidity of the bilayer would impact the gating of the channel. Different concentrations of cholesterol were tried to change the fluidity of the bilayer, but no changes in the gating behavior were observed. The composition of the
bilayer was also changed to incorporate photo-polymerizable lipids to decrease the fluidity. However these lipids proved to allow ionic current to leak through the membrane and provide too much noise to be useful.

6.2.4 Discussion of Viscosity Effects

The changes in solution viscosity yielded an observable change in the rate of channel closing, as shown in Figure 6.8. The change in the rate was a consequence of the additional work being done against the viscous fluid. However, since the system was persistently in the presence of a fluid, these studies could only make relative comparisons with the pure electrolyte solution.

Interestingly, the observations that the gating was restricted by the addition of the viscogen suggested that the channel gating was achieved by the movement of a physical element which restricted the flow of ions through the lumen of the channel. This is an important finding because no other investigations have been able to observe the mechanism of channel gating. Ion channel studies have verified the movement of a gating mechanism by measuring the gate current due to the movement of a charged domain under an applied electric field. Careful investigations with lysenin have not been able to detect such gate currents, so changing the electrolyte viscosity was used to verify the structure.

6.3 Temperature

6.3.1 Introduction

In Chapter 3 the open probability of the lysenin channels was shown to follow Boltzmann’s statistics in Equation 3.12 as:

$$P_{open} = \frac{1}{1 + e^{\frac{-\Delta E + qV}{k_B T}}}$$  (3.12)
For all previous analysis, the investigations were performed at room temperature (22°C). However, Equation 3.12 states that the open probability was temperature dependent. Consequently, changes in temperature shifted the open probability which altered the observed channel behavior. This section discusses the changes in the channels’ voltage response as the temperature of the system was changed. The nature of these investigations did not permit the temperature analysis of the channel to be conducted independently from the BLM or the electrolyte solution. Therefore, these two aspects were considered when evaluating the differences in the channel behavior for different temperatures.

Previous investigations with ion channels had provided evidence that the transitioning of a channel between states was a thermally driven process due to the energy barriers separating the states [20, 21, 29, 50, 97]. Therefore, the influences of temperature on lysenin channels was thought to influence the relative values of $E_o$ and $E_c$ as presented in Figure 3.4 by changes in the relative barrier height.

6.3.2 Methods

The temperature was regulated with a temperature controller designed for use with planer lipid bilayers as described in Chapter 2. The chamber was covered during the entire investigation to minimize evaporation which would increase the ion concentration in the solution. The temperature was monitored with a digital thermometer, and the system was allowed to equilibrate at each temperature before the voltage-induced gating was examined to ensure uniform temperature distribution throughout the chambers.
6.3.3 Temperature Dependence, $Q_{10}$

The dependence of temperature on a conductance channel was expressed by the $Q_{10}$ factor given in Equation 6.4.

$$Q_{10} = \left( \frac{I_2}{I_1} \right)^{10/(T_2-T_1)} \quad (6.4)$$

Where $I_2$ and $I_1$ were the currents through the channel at temperatures $T_2$ and $T_1$ respectively. The $Q_{10}$ provided the factor of the change in the channel conductivity over a temperature change of 10°C. It was used to compare the temperature sensitivity of ion channels, where higher $Q_{10}$ values denoted higher temperature sensitivity. Channels with a $Q_{10}$ in the range of 1.2-1.7 were not considered to be highly temperature dependent [4, 97]. The changes in current that yielded a $Q_{10}$ in this range were more likely resultant from changes in ionic mobility due to the temperature change, and indicated that the temperature does not play a significant role in the conformational changes of the protein leading to channel gating as compared to proteins with a higher $Q_{10}$ [20]. Using the open channel current at 10 mV for 10°C and 40°C, the $Q_{10}$ of lysenin channels was calculated to be 1.3. This finding qualitatively signifies that the temperature was not playing a significant role in the conformational changes in the protein and was not unexpected since changes in temperature did not induce channel gating independent of applied voltage.

6.3.4 Electrolyte and Open Channel Conductance

Before the effects of temperature on voltage-gating were examined, the role temperature played in the conductivity of the electrolyte solution was investigated. In the previous section, the $Q_{10}$ value was presented to be the result of changes in the electrolyte conductivity. This
section presents the reasons for the change in conductivity and establishes one of the underlying reasons for the observed changes in the I-V response.

The temperature studies were performed with 135 mM NaCl, 20mM HEPES electrolyte solution at pH = 7. The electrolyte conductivity was measured for increasing temperatures by warming the solution in a water bath, being sure to allow the sample temperature to equilibrate. The conductivity meter was calibrated to a 10 mS calibration solution at 25°C before the conductivities of the electrolyte were measured. The conductivity of the electrolyte was linear with temperature as indicated by Equation 6.5.

\[
\sigma(T) = \sigma_0 \left( 1 + \alpha (T - T_0) \right)
\]

Where \( \sigma_0 \) was a reference conductivity measured at temperature \( T_0 \) and \( \alpha \) was the temperature coefficient for NaCl. The temperature coefficient was calculated from two conductivities (\( \sigma_{T_1} \)) and (\( \sigma_{T_2} \)) measured at two temperatures (\( T_1 \)) and (\( T_2 \)) respectively.

\[
\alpha = \left( \frac{\sigma_{T_2} - \sigma_{T_1}}{\sigma_{T_1} (T_2 - T_1)} \right) \cdot 100
\]

The temperature coefficient was calculated to be 2.1 °C\(^{-1}\) and represented a 2.1% change in solution conductivity per °C. The average measured conductivities for different temperatures are shown in Figure 6.9. The error bars represent the standard deviations from the average conductivity.
Figure 6.9: Conductivity of 135 mM NaCl, 20 mM HEPES as a function of temperature.

As expected, the plot of the measured electrolyte conductivity shown in Figure 6.9 was linear with temperature. The best-fit line, shown in red, yielded a slope of $0.293 \pm 0.003 \, (\Omega \cdot m \, ^\circ C)^{-1}$ and an intercept at $7.407 \pm 0.065 \, (\Omega \cdot m)^{-1}$. From Equation 6.5, the theoretical slope was calculated to be 0.293 and the intercept was 7.43.

To determine the open channel conductance, the slope of the linear segment of the temperature variant I-V curves was measured at low positive voltages. Figure 6.10 shows the channel conductance for increasing temperatures.
Figure 6.10: The conductance of the open channel as function of temperature.

Similar to the solution conductivity, the channel conductance also had a linear relationship with temperature. This suggested that the temperature alone does not influence the conduction state of the channels. This finding was consistent with a model where temperature influenced the relative barrier height, but does not, on its own, shift the open probability. However, temperature did play a more significant role in the shift in open probability from an applied voltage.

6.3.5 Influence of Temperature on Voltage-Induced Gating

According to the model presented, increases in the temperature were expected to lower the relative barrier height between the potential wells allowing an easier transition between conduction states. This was observed during the applied voltage stimulation as a decreased critical voltage for increased in temperature. Figure 6.11a shows the I-V curves reflecting the lysenin channel response for changing temperatures. The larger current at higher temperatures was due to the increased electrolyte conductivity at higher temperatures as discussed previously.
Figure 6.11: (a) The I-V curves demonstrating channel inactivation for the temperatures indicated. (b) The I-V curves shown in (a) fit with Equation 3.14.

As the temperature of the system was increased from 10°C to 40°C, the channel inactivation occurred at successively lower voltages as predicted. The I-V curves were fit to Equation 3.14, shown in Figure 6.11b, to determine the values of $V_c$ and $\Delta E$ as a function of temperature. The relationship between critical voltage and $\Delta E$ with temperature is shown in Figure 6.12.

In Figure 6.12a, the plot of the critical voltages depicts the decreased critical voltages with increased temperature. Analysis of the plot suggested that continued increased temperatures exhibited diminished effects on the critical voltages. Between 10°C and 17°C, the temperature increased by 70% which corresponded to a 16% decrease in the critical voltage. Between 34°C and 40°C, the temperature increased by 18%, but the critical voltage decreased by 8%. The
critical voltage/temperature relationship implied that further increased temperatures caused the critical voltages to approach a constant value and that changes in the gating were no longer effected by temperature. However, the protein and BLM was not tolerant of these higher temperatures making testing such inferences impractical.

![Graph](image)

Figure 6.12: (a) The critical voltages from the I-V curves as a function of temperature. (b) Values of ΔE for increasing temperatures fit from Equation 3.14.

In Figure 6.12b, ΔE was found to decrease with increased temperature which verified that the temperature influenced the conformation of the protein in addition to the transition rates given by Equations 3.15 and 3.16. The influence of a phase transition of the lipid bilayer was experimentally excluded as a possible mechanism for channel gating. The capacitance of the lipid bilayer remained unchanged by temperature within the range investigated.

Chapter 4 introduced the hypothesis that the lag between the electrode voltage and the membrane voltage was expressed by a voltage difference (δV) given in Equation 5.2. Since increasing the temperature was found to lower the voltage required for channel state transitions, it was also expected to diminish the relative lag between the electrode voltage and the membrane voltage.
To best demonstrate the change in $\delta V$, the open probabilities from Equation 3.12 were extracted from the fit of I-V curves for channel inactivation and reactivation at different temperatures. Figure 6.13 shows the shift in $\delta V$ in the open probabilities of a channel population for increasing (black) and decreasing (red) voltage at the two extremes of 10°C and 40°C.

As predicted, increases in temperature caused the persistent voltage ($\delta V$) to decrease reducing the hysteresis behavior at higher temperatures. At 10°C, the lower temperature, shown in Figure 6.13a, was creating a greater difference between the open and closed state energies. Thus, a larger voltage difference was required to cause channel inactivation than for channels held at 40°C shown in Figure 6.13b where the higher temperature produced a smaller difference in state energies.

![Figure 6.13: Plots of the open probability functions for (a) 10°C and (b) 40°C. Inactivation curves are shown in black, and reactivation curves are shown in red.](image)

It was hypothesized in Section 4.5 that the difference in the inactivation and reactivation voltages decreased as the temperature approached $T_{\text{free}}$. It was shown in Equation 4.22 that difference in voltage was proportional to the change in temperature referenced from $T_{\text{free}}$.

$$\delta V \propto C - \sqrt{T}$$  \hspace{1cm} (4.22)
\( \delta V \) was calculated from hysteresis curves taken at different temperatures, and a plot of \( \delta V \) versus \( T \) is shown in Figure 6.14. The error bars shown are fitting errors from the inactivation and reactivation I-V curves. The points in Figure 6.14 were fit to Equation 4.22 to calculate a theoretical temperature where the hysteresis disappears. \( T_{\text{free}} \) was calculated to be 321.6 ± 0.2 K.

![Figure 6.14: A plot of the persistent voltage as a function of temperature.](image)

This analysis of the effects temperature on the voltage-induced gating and subsequent hysteresis reflected the model discussed previously. This channel behavior arose from the interaction between the channel and the lipid bilayer along with the electrolyte and environmental factors present in the system.

6.4 Summary

This chapter examined two distinct behaviors of lysenin channels and from those observations provided important insight into the characteristics of this protein. The first section revealed that the channel contained a gating mechanism that was at least partially exposed to the
solution. The hindrance that an increased viscosity provided to the gating indicated that the closure of the channel was attributed to a physical reorientation by some element of the protein in the presence of an applied electric field.

The observations made with changes in temperature were significant to understanding the energetics of the two-state channel system. The complexity of the transition between the open and closed states provided a challenge in isolating specific components of the system to determine exactly what mechanisms are involved.
CHAPTER 7: Conclusions

7.1 Discussion

This work revealed the fascinating properties of the pore-forming protein, lysenin. However, even though the investigations were limited to lysenin, this protein’s characteristics are representative of the functionality of many other protein channels and establishes a foundation for which could lead to further understanding of the mechanisms of ion-channel behaviors.

The voltage-induced gating exhibited by lysenin channels provided insight into structural elements of the protein channel and revealed that the orientation of an electric field with respect to the protein plays a critical role in the behavior of the channel. The interaction between an electric field and a voltage-gated ion channel has been previously established and thoroughly studied [30, 33, 38], but this dissertation emphasized the relationship among the channel protein, the electrolyte and the lipid membrane each of which acted collectively to produce the observed behavior. By investigating lysenin, the fundamental functionality of a voltage sensitive membrane transporter was established which allows for further understanding of analogous ion channels.

The studies presented here demonstrated that the fundamental mechanism of voltage-gating is subject to subtle variations in the gate charge, the orientation with respect to an electric field, or the lipids comprising the membrane. However, it was proven that the basic behavior of the protein was predictable with a model that incorporates the properties of the electrolyte solution and lipid membrane.

The most significant finding from this work was the role that the lipid membrane played in the function of the lysenin channel. Lipid membranes have been shown to have considerable
influence over the behavior of ion channels, but those studies focused on the physical interaction between the two or what can be categorized as a ligand-protein interaction [98]. This work demonstrated that the electrostatic interactions were not only important, but were hypothesized to be the foundation of additional behaviors as well.

The theory of the hysteresis behavior by lysenin channels developed a mechanism to explain the observation of the channels apparent ability to remember a previous state. This mechanism proposed that the dielectric properties of the lipid membrane were responsible for the asymmetry in the channel response to an external electric field. This theory suggested that elements of memory originate at the molecular level. The implications of this work can further research into the role of ion channels in memory disorders.

Recent investigations into the structure of lysenin have uncovered important characteristics that shed light onto the results of the work discussed here [72]. The protein structure takes on different orientations before and after interacting with sphingomyelin. That was explained by the rotation of part of the protein structure as shown in Figure 7.1a.

Upon examining the electrostatic surface potential [99], it was found that lysenin contains a positively charged region in the area that interacts with the headgroups of sphingomyelin depicted in blue in Figure 7.1b [100]. Further away, in the domain suspected to be part of the inside of the channel lumen, the protein has a negatively charged region shown in red in Figure 7.1b. Analysis of the published protein structure data yielded a protein length of approximately 9.7 nm. Given that individual proteins were assumed to span the lipid bilayer, a length on the order of 10 nm is reasonable.
Figure 7.1: (a) A cartoon representation of lysenin indicating the change in orientation due to the interaction with lipids. (b) The electrostatic surface potential representation of lysenin.

This study provided validation to the electrophysiological results presented here. It suggests that lysenin is oriented in the membrane in a way that a positive moving portion of the protein could respond to an external electric field as shown in Figure 7.2.

Figure 7.2: A possible orientation of two lysenin proteins and their response to an applied electric field to cause channel gating.

Given the distribution of charge in the protein shown in Figure 7.2, it is reasonable to speculate that the gating may be a result of the alignment of the protein’s dipole moment to the
applied electric field. Additionally, the interaction of the protein with the lipids suggested that the lipids would provide a mechanism to return the channel to the open state in the absence of an electric field. In this way, these new findings on lysenin’s structure agree with the conclusions in this work regarding the structure of lysenin.

7.2 Future Work

There remains much further work toward the complete characterization of lysenin channels. It is clear that this protein possesses great possibilities to model ion channel systems for further study. It lends itself for the study of ligand-induced gating which was not discussed here, but has been explored in much further detail elsewhere [9, 12].

While this study exclusively focused on the investigation of lysenin channels in a planar lipid bilayer membrane, it established the foundation for work in liposomes. The advancements made in understanding the mechanism of lysenin gating show promise in using it as a controllable nano-scale “valve” for transmembrane transport. Exploiting lysenin’s properties for use in a device opens a entire new avenue of possibilities for this unique protein.

Despite all of this work with investigating the interaction between a voltage-sensitive domain in the protein channel and an external electric field, a comprehensive picture of the lysenin channel remains incomplete. However, the crystal structure of the lysenin protein has recently been published [72]. By utilizing an electrostatic surface representation of the protein, depicted in Figure 7.3, charged domains can be identified to investigate the functionality of specific regions.
Figure 7.3: Electrostatic surface representation of a lysenin protein. The images (a), (b), (c), and (d) are the same protein image rotated by 90°.

Modification of those specific regions utilizing complex biological assays like recombinant expression will lead to identifying elements of the protein responsible for membrane insertion, pore formation, and voltage-induced gating. Complementing the techniques described here with advanced imaging such as TEM will result in a more complete picture, leading to further advancements in our understanding of these remarkable biological systems.
REFERENCES


77. The PyMOL Molecular Graphics System, Version 1.5 Schrödinger, LLC.


100. Jmol: an open-source Java viewer for chemical structures in 3D. http://www.jmol.org/
APPENDIX A: Description of research

Toxin Protein Has Ability to Remember

Researchers at the University of Arkansas have uncovered interesting memory behavior from an unexpected source. The protein Lysenin is a pore-forming toxin which was revealed to exhibit memory of recent events. “The protein has some other intriguing characteristics, but this is truly remarkable,” says Eric Krueger, a PhD student in the microEP program working with Professor Greg Salamo, “it is able to remember the previous environmental conditions.” The protein responds to voltage, but it can remember if a previous voltage was higher or lower.

Lysenin is a protein that is found in the earthworm *E. foetida* which is an earthworm commonly used for composting. “We don’t know what the worm uses it for, but it is thought to be part of their immune system,” remarks Krueger. The protein forms a channel in the lipid bilayer comprising the cell membrane. The channel causes the cell to die because it is no longer able to control what goes in or out. The channel-forming ability is why lysenin is considered a toxin. According to Krueger, if the cell membrane contains a lipid called sphingomyelin, then it is susceptible to be infected by lysenin. “For some reason, the cell membrane must contain sphingomyelin for lysenin to form a channel. Mammals have it [sphingomyelin] in their cells, so we have to take precautions when handling the protein,” warns Krueger.

Lysenin responds to a voltage by closing its channel, but simply reducing the voltage does not necessarily reopen it. Once closed, the voltage has to be reduced significantly to get it to reopen. If the voltage is increased again, the channel stays open until that closing voltage is reached again. Since the channel closes and reopens at different voltages, there is overlap where
the channel could be open or closed at the same voltage. “It sounds confusing, but basically whether a channel is open or closed at a given voltage depends on whether the previous voltage was higher or lower,” says Krueger.

![Figure A.1: A schematic of open and closed lysenin channels inserted in a lipid bilayer membrane.](image)

This discovery has implications beyond a simple earthworm protein. Krueger hopes that this work will provide insight into how our brain works. “Lysenin exhibits some similarities to the ion channels responsible for transmitting messages in our brains; maybe this will lead to new discoveries into the inner-workings of our brains.” This research has the potential to expose the fundamental mechanism of memory on a cellular scale. In doing so, it may provide insight into how we are able to store memories on the larger scale.
APPENDIX B: Newly created intellectual property

This appendix contains a list of the intellectual property created during the course of this research.

1. A method to control the pathway through a bilayer lipid membrane using lysenin channels.
2. A method to model and predict the hysteresis of lysenin channels due to an applied electric field.
3. A method to alter protein functionality by changing external variables.
4. A method to use lysenin channels as a sensor to detect changes in pH, lipid membrane composition, and temperature.
APPENDIX C: Potential patent and commercialization aspects

This section evaluates the information discussed in Appendix B to determine the patent or commercialization potential.

C.1 Potential Patentability

Lysenin is a naturally occurring protein harvested from an earthworm. The wild-type variety of this protein used extensively in this dissertation was purchased and used without further purification. Any intellectual property or potential patents on the methods of extraction, isolation, and purification of the wild-type protein from *Eisenia fetida* do not belong to the University of Arkansas.

1. Protein insertion into a lipid bilayer is a natural phenomena expressed by pore-forming toxins that cannot be patented. The control of the conduction pathway of a protein channel is a natural process and cannot be patented. However, using the lysenin channel as a controllable valve as discussed in Chapter 7 could be patented, but that work is not the scope of this dissertation.

2. A method of modeling and predicting the hysteresis of the macroscopic current cannot be patented. It is a process, but since it results in a scientific principle it is not eligible for patent protection defined by 35 U.S.C. § 101.

3. A method for altering protein functionality by changing its physical characteristics by an external stimuli such as pH and temperature is a natural phenomenon that cannot be patented under 35 U.S.C. § 101.
4. A method of using lysenin as a sensor for the detection of pH, lipid membrane composition, and temperature exploit the protein’s natural response to such stimuli. A patent could be obtained to protect its use as the bioactive material in a biosensor.

C.2 Commercialization Possibilities

1. Commercialization possibilities from the control of the channel pathway should be explored, but more experimental work is needed to examine where those possibilities exist. The extent of commercialization of this intellectual property is projected to consist of the pharmaceutical industry. However, further investigations into the role that lysenin may play for controlled drug delivery is beyond the scope of this dissertation.

2. While a method of modeling and predicting the hysteresis of the macroscopic current cannot be patented, the intellectual property should be protected. This work will be published in peer reviewed literature to ensure it remains available for public service.

3. Even though altering protein functionality by changing external stimuli cannot be patented, commercialization of this aspect remains possible. Possible patentability would protect its use as a biosensor, so the scope of commercialization would be associated with biosensing and bioinstrumentation.

4. Lysenin’s response to changes in pH, lipid membrane composition, and temperature could be used to develop a sensor for the detection of these variables. However, the fragility and limited lifetime of the membrane does not lend itself to market a reliable and robust sensor. The commercialization of this aspect is not advised.
C.3 Prior Disclosure

The voltage-induced gating, pH, temperature, and lipid membrane sensitivity have been published in the journals listed in Appendix G. The model was presented in a poster during the 4th Annual Nanotechnology for Health Care Conference in September 2012.

Prospective use in biosensing targeted for pH, lipid membrane composition, and temperature has not been previously disclosed.
APPENDIX D: Broader Impact

D.1 Applicability of research methods to other problems

The research methods described in this work has uncovered the mechanisms significant to the functionality of membrane transporters. This has provided a foundation for the study of other pore forming toxins and opens ion channels. The research has the potential to change future studies regarding the mechanisms of cellular memory.

D.2 Impact on US and global society

Lysenin’s resemblance to ion channels can help fuel research into the mechanisms of ion channel behavior. This can have impact in medical research in the investigation of ion channel related disorders as described in Chapter 1. The relative simplicity of using lysenin as compared to ion channels can help narrow possible avenues of research quickly and at lower cost.

D.3 Impact on the environment

Lysenin is considered a toxin in that it causes cell lysis through the mechanism of forming channels in the cell membrane. It has been reported that once lysenin is inserted into a membrane, it no longer poses a biological threat [73]. However, lysenin waste recovered from the experimental chambers is collected to be discarded as hazardous waste as per university hazardous waste disposal regulations.
APPENDIX F: Identification of all software used in research and thesis/dissertation

Computer #1

Model: Dell Precision T3400
Serial Number: DST4JJ1
Location: PHYS 129
Owner: Dr. Greg Salamo

Software #1

Name: Microsoft Office 2007
License #: 89396-707-0089972-65259
Purchased by: Dr. Greg Salamo

Software #2

Name: Origin 8
License #: GF3S4-9489-7603011
Purchased by: Dr. Greg Salamo

Software #3

Name: Endnote X
Purchased by: Dr. Greg Salamo

Software #4

Name: Symyx Draw 4.0
Purchased by: Freeware

Software #5

Name: pCLAMP 10.2
License #: 133833
Purchased by: Dr. Greg Salamo

Software #6

Name: Adobe Illustrator CS 5.1
Purchased by: University of Arkansas license

Software #7

Name: Microsoft Project 2007
Purchased by: College of Engineering site license

Computer #2

Model: Dell Optiplex 330
Serial Number: 88NGZD1
Location: NANO 241
Owner: Dr. Greg Salamo

Software #1

Name: pCLAMP 10.2
License #: 133833, requires hardware key
Purchased by: Dr. Greg Salamo
Computer #3

Model: Dell Optiplex 760
Serial Number: FHZPDK1
Location: Boise State University
Owner: Dr. Greg Salamo

Software #1
Name: pCLAMP 10.2
License #: 820664, requires hardware key
Purchased by: Dr. Greg Salamo

Computer #4

Model: Dell Latitude 810
Serial Number: JJWLZ61
Location: Personal Laptop
Owner: Eric Krueger

Software #1
Name: Microsoft Office XP
License #: 54186-640-1147967-17064
Purchased by: Eric Krueger
Software #2
Name: Symyx Draw 4.0
Purchased by: Freeware
APPENDIX G: Publications


APPENDIX H: The Ag/AgCl Electrodes

The electrodes are responsible for applying the voltage to the BLM and measuring the ionic current. The ions in the electrolyte solution are the charge carriers that produce the current through the lysenin channels. With Ag/AgCl electrodes as with any non-polarizing electrodes, there is a direct transfer of charge between the electrode and solution [80]. This appendix offers a brief explanation as to the electrode/electrolyte interaction.

The electrolyte solution in this model consisted of NaCl in deionized water. As shown in Figure H.1, the electrodes were immersed in the electrolyte and a voltage was applied. To understand the flow of current, it is helpful to follow the movement of charge through the system.

![Diagram of Ag/AgCl electrodes in electrolyte solution](image)

Figure H.1: A diagram of the Ag/AgCl electrodes in electrolyte solution.

The electrodes consisted of a Ag wire that had been chlorinated to develop a coating of AgCl over the outside. AgCl is an insoluble salt in aqueous solutions. Electrons moved along the
Ag wire and converted the AgCl to Ag atoms and Cl\(^-\) ions. The Cl\(^-\) ions entered the solution where they diffused along the electric field towards the positive electrode.

At the other electrode, Cl\(^-\) ions in solution reacted with any Ag atoms to become AgCl and transferred the electron to the Ag wire. Through this mechanism, a complete circuit was achieved to allow current to flow.
APPENDIX I: Derivations

I.1 Probability and Energy of the Two-State System

The state of a system \(a\) can be designated by \(a_1, a_2 \ldots n\) where \(1, 2 \ldots n\) is the state. Assume that the state of the system is dependent on the energy where \(E_1\) is the energy of \(a_1\), \(E_2\) is the energy of \(a_2\), etc. The ratio of the system states can be written as:

\[
\frac{a_2}{a_1} = f(E_1, E_2) \quad (I.1)
\]

If the reference point of the energies is defined to be \(E_2\), then the function becomes:

\[
f(E_1, E_2) = f(E_1 - E_2, 0) \quad (I.2)
\]

The ratio of states \(a_1\) and \(a_2\) can then be written as:

\[
\frac{a_2}{a_1} = f(E_1 - E_2) \quad (I.3)
\]

In general, the ratio between any two states must follow the same form.

\[
\frac{a_3}{a_1} = f(E_1 - E_3) \quad (I.4)
\]

\[
\frac{a_3}{a_2} = f(E_2 - E_3)
\]

Therefore we can write:

\[
\frac{a_3}{a_1} = \frac{a_3}{a_2} \cdot \frac{a_2}{a_1} \quad (I.5)
\]

Combining Equations I.3 and I.4 with the relationship in Equation I.5 we get:

\[
f(E_1 - E_3) = f(E_1 - E_2)f(E_2 - E_3) \quad (I.6)
\]

This is true if the function \(f\) takes the form:

\[
f(E) = e^{\beta E} \quad (I.7)
\]
Therefore, Equation I.6 can be written as:

\[ e^{\beta(E_1 - E_3)} = e^{\beta(E_1 - E_2)} e^{\beta(E_2 - E_3)} \] (I.8)

In general, Equation I.3 can now be written as:

\[ \frac{a_n}{a_m} = e^{\beta(E_m - E_n)} \] (I.9)

Where \( \beta = \frac{1}{k_B T} \). From the properties of exponentials we get that the energy of any state \( n \) is:

\[ a_n = C e^{-\beta E_n} \] (I.10)

Where \( C \) is a constant. To solve for \( C \) we find the sum of both sides as:

\[ \sum_n a_n = \sum_n C e^{-\beta E_n} \]

\[ C = \frac{\sum_n a_n}{\sum_n e^{-\beta E_n}} \] (I.11)

The sum over \( a_n \) is the total number of systems, which is a constant (\( \mathcal{A} \)). Substituting back into Equation I.10 we get:

\[ a_n = \frac{\mathcal{A}}{\sum_n e^{-\beta E_n}} e^{-\beta E_n} \] (I.12)

The value \( \frac{a_n}{\mathcal{A}} \) is the fraction of the total number of systems in state \( a_n \) which have energy \( E_n \). This is defined as the probability \( (P_n) \) analogous to Equation I.3

\[ P_n = \frac{a_n}{\mathcal{A}} = \frac{e^{-\beta E_n}}{\sum_n e^{-\beta E_n}} \] (I.13)
Therefore, in the case of the two-state system that lysenin channels exhibit, the probability of states 1 and 2 can be written as:

\[ P_1 = \frac{e^{-\beta E_1}}{e^{-\beta E_1} + e^{-\beta E_2}} \]

\[ P_2 = \frac{e^{-\beta E_2}}{e^{-\beta E_1} + e^{-\beta E_2}} \]  

(I.14)
I.2 Derivation of the Time Dependent Open Probability and Current

Lysenin channels exist in two discrete states which transition between the two at a rate defined by the rate constants for opening \((k_o)\) and closing \((k_c)\).

\[
\frac{k_c}{k_o} \\
\text{Open} \rightleftharpoons \text{Closed}
\]

The total number of channels is constant, and is the sum of the number of open channels \((N_o)\) and closed channels \((N_c)\).

\[
N = N_o + N_c \tag{I.16}
\]

The probability of a channel to be in the open state at equilibrium is defined as:

\[
P_{\text{open}} = \frac{N_o}{N} \tag{I.17}
\]

Since the observation of lysenin channels’ state is through the ionic current which is only conducted through open channels, the derivation that follows will be for open channels. The number of open channels changes in time as:

\[
\frac{dN_o}{dt} = -N_0 k_c - N_c k_o \tag{I.18}
\]

Combining Equation I.16 and Equation I.18 to substitute for \(N_c\):

\[
\frac{dN_o}{dt} = -N_0 (k_c + k_o) - N k_o \tag{I.19}
\]

Separating the time component:

\[
\frac{dN_o}{N_0 (k_c + k_o) - N k_o} = -dt \tag{I.20}
\]

Multiply both sides by \((k_c + k_o)\):

\[
\frac{dN_o}{N_0 (k_c + k_o) - N k_o} (k_c + k_o) = - (k_c + k_o) dt \tag{I.21}
\]
The limits of integration are defined as all of the channels are open, \( N_0 = N \), when \( t = 0 \).

\[
\int_{N}^{N_0} \frac{(k_c + k_o)}{N_0(k_c + k_o) - Nk_o} dN_o = -\int_0^t (k_c + k_o) dt
\]  
(I.22)

\[
\ln(N_0(k_c + k_o) - Nk_o) - \ln(N(k_c + k_o) - Nk_o) = -(k_c + k_o)t
\]  
(I.23)

\[
\ln(N_0(k_c + k_o) - Nk_o) - \ln(Nk_c) = -(k_c + k_o)t
\]  
(I.24)

\[
\ln\left(\frac{N_0(k_c + k_o) - Nk_o}{Nk_c}\right) = -(k_c + k_o)t
\]  
(I.25)

Solving for \( N_0 \):

\[
\frac{N_0(k_c + k_o) - Nk_o}{Nk_c} = e^{-(k_c+k_o)t}
\]  
(I.26)

\[
\frac{N_0(k_c + k_o)}{Nk_c} - \frac{Nk_o}{Nk_c} = e^{-(k_c+k_o)t}
\]  
(I.27)

\[
\frac{N_0(k_c + k_o)}{Nk_c} = e^{-(k_c+k_o)t} + \frac{k_o}{k_c}
\]  
(I.28)

\[
N_0(t) = N \frac{k_c}{(k_c + k_o)} e^{-(k_c+k_o)t} + N \frac{k_o}{(k_c + k_o)}
\]  
(I.29)

The numerators in Equation I.29 are different in the time dependent term and in the independent term. To obtain the open probabilities, first recall the equilibrium relationship between the number of open and closed channels and the rate constants from Equation 3.5.
\[
\frac{N_c}{N_o} = \frac{k_c}{k_o}
\]

The open probability from Equation \( I.17 \) becomes:

\[
P_o = \frac{k_o}{(k_c + k_o)} \quad (I.30)
\]

And the closed probability can be defined as:

\[
P_c = 1 - P_o = \frac{k_c}{(k_c + k_o)} \quad (I.31)
\]

Equations \( I.30 \) and \( I.31 \) can be combined with Equation \( I.29 \) to become:

\[
N_o(t) = N(1 - P_o)e^{-(k_c+k_o)t} + NP_o \quad (I.32)
\]

A time constant \( (\tau) \) can be defined as:

\[
\tau = \frac{1}{(k_c + k_o)} \quad (I.33)
\]

Equation \( I.32 \) then becomes:

\[
N_o(t) = N(1 - P_o)e^{-\frac{t}{\tau}} + NP_o \quad (I.34)
\]

The number of open channels as a function of time is a result in the time dependent open probability, which can simply be written as:

\[
\frac{N_o(t)}{N} = P_o(t) = (1 - P_o)e^{-\frac{t}{\tau}} + P_o \quad (I.35)
\]

In a more general sense, Equation \( I.35 \) should be interpreted as being constructed of the initial \( (t = 0) \) open probability and the equilibrium \( (t = \infty) \) open probability. In the case of the step voltage closing, the initial probability was 1 since the applied voltage step started at \( V = 0 \) mV. Therefore Equation \( I.35 \) can be rewritten as:

\[
P_o(t) = (P_o(t=0) - P_o(t=\infty))e^{-\frac{t}{\tau}} + P_o(t=\infty) \quad (I.36)
\]
In lysenin experiments the measurements obtained to observe the state of the channels is the ionic current conducted by the open channels. The current corresponds to the time dependent open probability as the time dependent current demonstrated in Figure 3.10 and Figure 3.11 in Chapter 3. Similar to the explanation for the time dependent probability in the case of a positive step voltage, the observed current for a constant applied voltage is given as:

\[ I(t) = VgNP_o(t) \] (I.37)

Where \( V \) is the applied voltage and \( g \) is the single channel conductance. At \( t = 0 \), all of the channels were open \( (P_{o,t=0} = 1) \) so the current is:

\[ I(0) = VgNP_o(0) \]

\[ I(0) = VgN = I_{max} \] (I.38)

At \( t = 0 \), the initial current is at its maximum value. Conversely, at \( t = \infty \), the current is at its minimum given as:

\[ I(\infty) = VgNP_o(\infty) = I_{min} \] (I.39)

Equation I.36 can be restated in terms of current using Equations I.37 and I.38.

\[ I(t) = (I_{max} - I_{min})e^{-\frac{t}{\tau}} + I_{min} \] (I.40)