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Functional and Structural Studies of the Human Voltage-Gated Proton Channel

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FUNCTIONAL AND STRUCTURAL STUDIES OF THE HUMAN VOLTAGE-GATED PROTON CHANNEL

A Thesis Presented to the Faculty of
The Rockefeller University
in Partial Fulfillment of the Requirements for
the degree of Doctor of Philosophy

by
James Anthony Letts
June 2014
The activity of voltage-gated cation channels underlies the action potentials that allow for neuronal signaling and muscle contraction. The canonical family of voltage-gated K⁺, Na⁺ and Ca²⁺ channels has been the subject of extensive electrophysiological, biophysical, genetic, biochemical and structural characterization since the 1950s. These channels all share a conserved six-transmembrane helix topology (S1-S6) in which the first four transmembrane helices (S1-S4) form the regulatory voltage-sensor domain and the last two transmembrane helices (S5 and S6) comprise the ion-conducting pore domain. It was thought that all voltage-gated cation channels shared this conserved domain architecture. However, this scheme was challenged by the discovery of the gene for the voltage-gated H⁺ channel. This voltage-gated cation channel has a four transmembrane helix topology that is homologous to the voltage-sensor domain of the canonical voltage-gated cation channels alone, without a separate pore domain.

In this thesis, I present my work, which constitutes the first ever biochemical characterization of the human voltage-gated H⁺ channel (hHᵥ1). First, I demonstrate by site-specific cross-linking that hHᵥ1 is a dimer in the membrane and define the oligomerization interface. Then, by developing methods for the heterologous expression, purification and reconstitution of hHᵥ1, I establish that the four transmembrane helix
voltage-sensor-domain-like putative channel protein is in fact responsible for H⁺ conduction. Next, I present my work on the structural characterization of hHν1 by X-ray crystallography. I solved a low-resolution structure of a chimeric voltage-gated proton channel but then demonstrated that although this channel is functional in a membrane, the conformation seen in the crystal is non-native. Finally, I present my work on the analysis of hHν1 by solution state NMR in detergent micelles. This technique allowed us to define the secondary structure of the channel for the first time but full three-dimensional structural characterization was determined to be unfeasible. From these studies, I conclude that the Hν channel structure is dependent on the constraints imposed by the lipid bilayer and is destabilized upon detergent solubilization. Future structural studies of Hν channels will have to focus on channels imbedded within a membrane-like environment.
To my parents, my brothers and my darling wife.
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CHAPTER 1: INTRODUCTION

In order to support the highly complex processes required for life, cells must be able to isolate desired components such as ions, small-molecule nutrients and biological macromolecules and precisely control their concentrations such that the chemistry of life, which derives from their interactions, can occur. This problem was solved in part by the evolution of the cellular membrane; an oily barrier composed of a bilayer of amphipathic phospholipids, which envelopes and defines the cell. Unlike the aqueous medium inside and surrounding cells, which allows for the free diffusion and interaction of biological molecules and ions, the membrane interior excludes water and hence creates an energetic barrier for passage of hydrophilic components. In this way, the membrane solves the problem of keeping the cells’ contents from diffusing away, allowing for the chemistry of life to occur.

However, the existence of the membrane creates a new problem: it blocks the release of waste products from inside the cell and the uptake of nutrients from outside. The evolution of “membrane proteins” that express hydrophobic exteriors and therefore are able to insert and reside within the cellular membrane, allowed cells to fully exploit
the membrane’s properties. Indeed, some membrane proteins have evolved to catalyze the specific conduction or transport of hydrophilic ions and molecules across the membrane, thus helping the cell gain precise control over its contents.

Another property of the cellular membrane that cells have evolved to utilize is its ability to separate electrical charge, i.e. its capacitance. Cells can store energy across the membrane by controlling the relative concentrations of ions inside and outside the cell. This energy manifests itself as an electrical potential across the membrane, which the cell can then use to do work. In most cells, this work is performed locally on a specific class of voltage-sensitive ion channels or enzymes, allowing for the fast transmission of signals along the cell membrane. Moreover, in certain organisms, specialized electrogenic organs have evolved that use biologically generated electric fields from an organized array of cells to detect the electric fields of other organisms, a process known as electroreception. In extreme cases, such as the electric knifefish (a.k.a. electric eel) and the electric rays, biological electricity is used in hunting to stun or even kill prey.

In this chapter, I will discuss how cells generate and control electrical potentials across the membrane using ion channels and transporters. I will introduce the canonical voltage-gated ion channels and how cells can use them to directly tie the membrane potential to membrane permeability. Then, I will introduce the biophysics of the voltage-gated proton channel and discuss its diverse physiological roles. Finally, I will discuss the motivation and aims for my research on the human voltage-gated proton channel.
1.1 Generation and Control of Cellular Electricity

1.1.1 Channeling Ions – the charge carriers of bioelectricity

The inorganic ions Na\(^+\), K\(^+\), Ca\(^{2+}\) and Cl\(^-\) are the major charge carriers used to generate electrical potentials across the membranes of cells (Hille 2001). The relative abundance of these ions on either side of the membrane is regulated by the activity of two classes of membrane proteins: ion channels and ion transporters (a.k.a. ion pumps).

Ion channels allow only for the passive diffusion of ions down their electrochemical gradient and are defined (and named) by two major properties: selectivity and gating. Selectivity refers to the fact that different channels show a preference for the conduction of specific ionic species. For example, a K\(^+\) channel allows for the easy passage of K\(^+\) ions across the membrane but not the easy passage of Na\(^+\) ions. Gating refers to the ability of the channels to open and close, that is, to transition between conductive and non-conductive states. The stimulus that causes a channel to gate varies widely between channels. Some channels are gated by the binding of ligands (for example the ATP-gated K\(^+\) channel, K\(_{ATP}\)), others are gated by the transmembrane potential (for example the voltage-gated K\(^+\) channel, K\(_V\)), others, by the mechanical state of the membrane itself (e.g. tension) and others, by environmental cues such as temperature and light (Hille 2001). Many channels are gated to varying degrees by the combination of different stimuli, for example the Slo1 K\(^+\) channel is gated both by transmembrane voltage and by binding of intracellular Ca\(^{2+}\) ions (Barrett et al. 1982).

Ion pumps use cellular energy (usually in the form of ATP) to pump ions against their concentration gradient (Albers 1967). For example, the Na\(^+\) K\(^+\) ATPase pumps three
Na\(^+\) ions out of the cell while at the same time pumping two K\(^+\) ions into the cell and hydrolyzing an ATP molecule to form ADP and inorganic phosphate (Post et al. 1972). Since the number of ions pumped in each direction is uneven and the charge on each ion is the same, this pumping cycle is electrogenic. Thus, the Na\(^+\) K\(^+\) ATPase separates charge across the membrane, thereby charging the membrane capacitor and contributing to the membrane potential (Albers 1967). In the next section, I will describe how the activity of ion channels and pumps working in concert can be used to control the electrical potential of the cell.

1.1.2 Electro-diffusion – how the membrane voltage is set

Ion pumps consume cellular energy to pump ions against their concentration gradients and thereby establish an uneven distribution of ions across the membrane (Albers 1967). For example, in mammalian skeletal muscle the distribution of ions is such that [Na\(^+\)] is high outside relative to inside, [K\(^+\)] is low outside relative to inside, [Ca\(^{2+}\)] is high outside relative to inside and [Cl\(^-\)] is high outside relative to inside (Table 1.1; Hille 2001).

Because each ion carries a charge, the free diffusion of the ions across the semi-permeable membrane does not depend solely on the concentration difference and does not go to equilibrium when the concentration of the specific ionic species reaches the same value on either side of the membrane (Nernst 1888; Nernst 1889; Hille 2001). For example, diffusion of a single K\(^+\) ion down its concentration gradient removes a positive charge from the inside of the cell and adds a positive charge to the outside. Therefore, diffusion of ions across the membrane creates an electrical potential resulting in a force opposing the further diffusion of similarly charged ions. Equilibrium is reached when the
strength of the electrical potential results in balanced diffusion, such that there is no net flux of ions across the membrane. The electrical potential at which this balance is reached is known as the Nernst equilibrium potential ($E_s$) and is calculated for each ionic species (S) using the Nernst equation (equation 1.1), where $R$ is the gas constant, $T$ is the temperature, $z_s$ is the charge on the ionic species, $F$ is Faraday’s constant, $[S]_o$ is the external concentration of the ion and $[S]_i$ is the internal concentration (Nernst 1888). The Nernst equilibrium potentials for each of the four major biologically important inorganic ionic species in muscle cells at $37^\circ$C are shown in Table 1.1 (Hille 2001).

$$E_s = \frac{RT}{z_sF} \ln \frac{[S]_o}{[S]_i} \quad \{1.1\}$$

<table>
<thead>
<tr>
<th>Ion</th>
<th>Extracellular Concentration (mM)</th>
<th>Intracellular Concentration (mM)</th>
<th>$\frac{[S]_o}{[S]_i}$</th>
<th>Equilibrium Potential$^a$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^+$</td>
<td>145</td>
<td>12</td>
<td>12</td>
<td>+67</td>
</tr>
<tr>
<td>K$^+$</td>
<td>4</td>
<td>155</td>
<td>0.026</td>
<td>-98</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>1.5</td>
<td>100 nM</td>
<td>15,000</td>
<td>+129</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td>123</td>
<td>4.2$^b$</td>
<td>29$^b$</td>
<td>-90$^b$</td>
</tr>
<tr>
<td>H$^+$ $^c$</td>
<td>$4 \times 10^{-5}$</td>
<td>$1 \times 10^{-3}$</td>
<td>0.04</td>
<td>-86</td>
</tr>
</tbody>
</table>

$^a$Calculated from equation 1.1 at $37^\circ$C

$^b$Calculated assuming a -90 mV resting potential for the muscle membrane and that Cl$^-$ is at equilibrium at rest.

$^c$Values from Carter et al. 1967
Because the cell membrane separates many different ionic species, the reversal potential \( (E_{\text{rev}}) \) at which there is no net ion flux across the membrane is not found at the Nernst equilibrium potential of a single ion, but at a weighted mean of all the Nernst potentials (Goldman 1943; Hodgkin & Katz 1949). The different Nernst potentials are weighted by the relative permeability of the different ions according to the Goldman-Hodgkin-Katz voltage equation, which if K\(^+\), Na\(^+\) and Cl\(^-\) are the permeant ions gives equation 1.2, where \( P_S \) is the permeability of the membrane to the specific ionic species \( (S = K^+, Na^+, Cl^-) \) and all other terms are defined as in equation 1.1 (Goldman 1943; Hodgkin & Katz 1949; Hille 2001).

\[
E_{\text{rev}} = \frac{RT}{F} \ln \frac{P_{K^+} [K^+]_o + P_{Na^+} [Na^+]_o + P_{Cl^-} [Cl^-]_o}{P_{K^+} [K^+]_i + P_{Na^+} [Na^+]_i + P_{Cl^-} [Cl^-]_i} \quad \{1.2\}
\]

Equation 1.2 holds only in the simplified case in which there are no active electrogenic pumps in the cell and all of the permeant ions carry the same absolute value of charge (the equation takes an alternate form when divalent cations such as Ca\(^{2+}\) are taken into account; Hille 2001). Although \( P_S \) is defined as the rate of flux of ion S across the membrane (in units of cm/s), it is proportional (though non-linearly) to the conductance of ion S across the membrane \( (g_S, \text{with units of Siemens} [\text{kg}^{-1}\cdot\text{m}^{-2}\cdot\text{s}^{-3}\cdot\text{A}^{2}] ) \), which is a function of the number of open ion channels that can conduct ion S and the driving force for the conduction of that ion (Hodgkin & Huxley 1952c; Hille 2001). If the membrane is only permeable to a single ionic species, equation 1.2 simplifies to the Nernst equilibrium potential for that ion (equation 1.1).
In the average resting cell, the presence of inward rectifying K$^+$ channels makes the membrane far more permeable to K$^+$ than to any other ionic species; therefore, $E_{\text{rev}}$ is close to $E_K$ (approximately -60 to -90 mV, see Table 1.1; Hille 2001). Through changes in the permeability of the membrane to the different ionic species (i.e. opening and closing of K$^+$ and Na$^+$ ion channels), the cell can control its $E_{\text{rev}}$, allowing it to set the membrane potential to any value between $E_K \approx -98$ mV and $E_{\text{Na}} \approx +67$ mV (Table 2; Goldman 1943; Hodgkin & Katz 1949; Hille 2001). Voltage-gated cation channels, whose open probability is a function of the membrane potential, directly tie the permeability of the membrane to K$^+$, Na$^+$ and Ca$^{2+}$ to the membrane potential, which allows for the rapid propagation of electrical signals across the membrane known as action potentials (Hodgkin & Huxley 1952a).

1.2 Voltage-Gated Ion Channels and the Voltage Sensor Domain

1.2.1 Discovery and common features of voltage-gated cation channels

Hodgkin and Huxley first described voltage-gated cation currents in their classical studies on the action potentials of the giant squid axon in the 1950s (Hodgkin & Huxley 1952c; Hodgkin & Huxley 1952b; Hodgkin & Huxley 1952d; Hodgkin & Huxley 1952a). As their name suggests, the opening and closing (i.e. gating) of the voltage-gated cation channels is strongly dependent on the transmembrane potential. It wasn’t until the 1980s that the first genes of a voltage-gated Na$^+$ ($Na_v$) channel and a voltage-gated K$^+$ ($K_v$) channel were identified and cloned (Noda et al. 1984; Tempel et al. 1987). These
sequences were then followed by the sequences of many more voltage-gated Na\(^+\), K\(^+\) and Ca\(^{2+}\) channels from many different species (for a review see Yu & Catterall 2004).

Analysis of these sequences identified a simple pattern: \(K_v\) channels were made up from a short polypeptide subunit, whereas, \(Na_v\) and \(Ca_v\) channels were made up of a longer polypeptide containing four “\(K_v\)-channel like” domains (Rehm & Tempel 1991; Guy & Durell 1994). Each one of the subunits or domains shared a conserved six-transmembrane-helix (6-TM) architecture (S1-S6) with a conserved series of positively charged arginine or lysine amino acid residues along the fourth transmembrane helix (S4), as well as conserved negatively charged residues on (S1 and S2; Fig 1.1; Rehm & Tempel 1991; Guy & Durell 1994; Yu & Catterall 2004; Bezanilla 2000). For the \(K_v\) channels, it was shown that the last two transmembrane helices (S5 and S6) comprise the pore of the channel (Fig. 1.1; Mackinnon & Miller 1989; Mackinnon et al. 1990; Mackinnon & Yellen 1990; Yellen et al. 1991). The discrepancy between the length of the \(K_v\) channel and \(Na_v/Ca_v\) channel genes was resolved when it was found that four identical \(K_v\) channel subunits come together in the membrane to form a four-fold symmetric tetramer (Mackinnon 1991). Based on sequence analysis, the first four transmembrane helices (S1-S4) were proposed to fold into a separate domain that was thought to be responsible for voltage sensing, and hence was coined the voltage-sensor domain (VSD; Fig. 1.1; Greenblatt et al. 1985; Nelson et al. 1999).
Fig. 1.1 Topology of voltage-gated channels

(A) Schematic representation of the six-transmembrane helix topology of $K_v$ channels which become concatenated to form the topology of the $Na_v$ or $Ca_v$ channels (B). The helices that comprise the voltage-sensor domain (S1-S4) in each domain are shown in orange while the helices that comprise the pore (S5 and S6) are shown in brown. The conserved gating-charge residues on S4 are indicated by “+” signs. Horizontal black lines denote the membrane boundaries (cytoplasm side down).
One of the most well characterized families of voltage-gated cation channels is the voltage-gated K⁺ channel family Kᵥ1.1 (for a review see Fedida & Hesketh 2001). This channel is closed at hyperpolarized membrane potentials (negative inside relative to outside) and opens upon depolarization (Fig. 1.2). By measuring the instantaneous current-voltage relation from the tail currents and plotting the normalized current ($I/I_{\text{max}}$) as a function of transmembrane potential ($V$), it is possible to get an estimate of the open probability of the channel at the different values of $V$ (Fig. 1.2; Hodgkin & Huxley 1952c; Hille 2001). This normalized current-voltage (IV) curve can be modeled by the two-state Boltzmann function shown in equation 1.3, where $z$ is the effective gating charge on the channel (see below), $V_{\text{mid}}$ is the voltage at which half the channels are open and $F$, $R$, and $T$ are defined as in equation 1.1.

\[
\frac{I}{I_{\text{max}}} = \frac{1}{1 + \exp\left(-\frac{zF}{RT}(V - V_{\text{mid}})\right)} \tag{1.3}
\]

As this equation shows, at $V$ greater than $V_{\text{mid}}$ the exponential term approaches zero and the ratio $I/I_{\text{max}}$ goes to one, at $V$ less than $V_{\text{mid}}$ the exponential term approaches infinity and $I/I_{\text{max}}$ goes to zero, at $V$ equals $V_{\text{mid}}$ the exponential term equals one and $I/I_{\text{max}}$ equals 0.5.
In order to sense and undergo conformational changes in response to changes in the transmembrane voltage, the channels must contain “gating charges” within the membrane interior, which move in response to changes in the transmembrane potential, resulting in channel gating (Hodgkin & Huxley 1952a). Gating charges have been directly observed during the gating of voltage-gated channels (Armstrong & Bezanilla 1973; Taglialatela & Stefani 1993; Perozo et al. 1992; Perozo et al. 1993). As equation 1.3 shows, this gating charge (z) is a scalar multiplier of the voltage difference (V-\(V_{\text{mid}}\)). Therefore, the gating charge modifies the steepness at which changes in voltage near \(V_{\text{mid}}\) alter the I/I_{\text{max}} ratio: the larger the gating charge, the smaller the voltage difference (V-\(V_{\text{mid}}\)) is required to bring I/I_{\text{max}} to one or zero. These gating charges were measured to be 12 to 14 elementary charges per channel (3.0 to 3.5 per subunit) and identified to be the
conserved positively charged amino acid residues on the S4 helix with contributions from a conserved negatively charged residue on S2 (Seoh et al. 1996; Aggarwal & Mackinnon 1996).

Although these charges are required for the proper functioning of voltage-gated cation channels, due to the low electrical permittivity of the membrane interior, charges are unstable within its depths (Yaroshchuk 2000). How voltage-gated channels circumvent this energetic barrier and stabilize the gating-charge arginines in the membrane was not fully understood until the elucidation of the eukaryotic Kᵥ channel structures (Long et al. 2005; Long et al. 2007).

1.2.2 Structure of the Kᵥ channel – the VSD revealed

The first structures of the prokaryotic Kᵥ channel KᵥAP established the domain arrangement of the channel’s fold (Jiang et al. 2003a). As was previously demonstrated, the Kᵥ channel was seen to be a four-fold symmetric tetramer with the tetramerization interface completely comprised of the last two transmembrane helices (S5 and S6) (Mackinnon 1991; Jiang et al. 2003a). The S5 and S6 helices from each subunit come together to form a central pore, with the ion conduction pathway down the central four-fold symmetry axis (Fig. 1.3A) (Jiang et al. 2003a). This pore structure was nearly identical to the structures of the non-voltage-gated K⁺ channels that had previously been solved (Doyle et al. 1998; Zhou et al. 2001; Jiang et al. 2002).
Fig. 1.3 Kᵥ channel Structure

(A) The pore domain of the Kᵥ1.2 channel shown in stick representation surrounded by translucent atom space filling spheres with carbon grey, nitrogen blue, oxygen red and sulfur yellow. VSDs have been removed for clarity (Long et al. 2007). The surface of the pore is shown in blue drawn with the program HOLLOW (Ho & Gruswitz 2008). (B) The Kᵥ channel tetramer looking down from the extracellular medium showing the peripheral arrangement of VSDs around the central pore domain, each subunit is colored differently with each α-helix represented as a cylinder (Long et al. 2007).
The first four transmembrane helices (S1-S4) arrange themselves around the periphery of the pore and form the VSD, which is responsible for voltage dependent conformational changes (Fig. 1.3B) (Greenblatt et al. 1985; Caprini et al. 2001). Although it was demonstrated that the conformation of the VSD seen in the full-length KvAP structure was in a non-native conformation (Jiang et al. 2003a; Lee et al. 2005; Long et al. 2005; Long et al. 2007), the structure did allow for the identification of the functionally important S3-S4 helix-turn-helix motif coined the “voltage-sensor paddle” (Jiang et al. 2003a). It has subsequently been shown that this motif is found in all VSD containing proteins and that it can be swapped between different VSDs and still result in functional voltage-gated channels (Alabi et al. 2007). Structures of the isolated VSD from KvAP and, later, the structure of eukaryotic Kv channels revealed the native four-helix bundle fold of the VSD (Fig. 1.4; Jiang et al. 2003a; Long et al. 2005; Long et al. 2007). These structures allowed the first understanding of how the gating-charge arginines are stabilized within the membrane and were later followed by the structures of prokaryotic Na channels (Payandeh et al. 2011; Zhang et al. 2012; Payandeh et al. 2012).
Fig. 1.4 The VSD structure from the K\textsubscript{1,2-2.1} Paddle Chimera

Structure of the VSD is shown in ribbon representation with the side chains of charged amino acid residues within the transmembrane region shown as sticks with carbon grey, nitrogen blue and oxygen red (Long \textit{et al.} 2007). The side chain of F233 is shown in blue. The S4 gating-charge residues are numbered according to the equivalent positions along the S4 of Shaker (Tempel \textit{et al.} 1987). Horizontal black lines denote the approximate membrane boundaries (cytoplasm side down). The sites of charge compensation within the membrane are highlighted by pink circles.
Because of the large energy cost of burying a charge in the low permittivity medium of the membrane interior, membrane proteins have evolved to minimize the number of charged amino acid side chains within their transmembrane segments (Eisenberg et al. 1984). However, if —such as in the case of a VSD— the presence of a charged residue in the membrane is mechanistically required, then compensation for the charge must be provided by the protein structure. In many cases, this compensation is provided by the presence of oppositely charged residues protruding from other parts of the protein. Charge compensation of this type can readily be seen in the structures of VSDs. As shown in Fig. 1.4, all of the positively charged S4 gating charges that are buried within the hydrophobic membrane are paired with a conserved negatively charged residue from one of the other transmembrane helices (Glu183 on S1, Glu226 and Glu236 on S2 and Asp259 on S3a). This pairing stabilizes the charges in the membrane, hence stabilizing the structure of the VSD itself.

1.2.3 Mechanism of voltage sensing – the charge transfer center

The internal charge-compensation site occupied by K5 in Fig. 1.4 is known as the charge transfer center (Tao et al. 2010). This site differs from the external charge-compensation sites by the presence of two negatively charged side chains and the proximity to the “phenylalanine gap” (represented by the blue side chain of F233 in Fig. 1.4). This phenylalanine gap forms a hydrophobic barrier that separates the external and internal charge compensation clusters and is considered the most constrictive point within the VSD structure (Fig. 1.3; Tao et al. 2010; Starace & Bezanilla 2004).
The structure shown in Fig. 1.4 is the depolarized conformation of the Kv1.2-2.1 paddle chimera VSD (Long et al. 2007). It is thought that, during gating, the S4 gating charge residues that occupy each of the charge compensation sites changes. For example, in the hyperpolarized state immediately preceding the depolarized state, R2 would be in the external charge compensation site occupied by R3 in Fig. 1.4, R3 would be in the site occupied by R4 and R4 will be in the internal charge transfer center occupied by K5. This coordinated movement between the sites would allow for sustained charge compensation throughout the gating conformational changes of the VSD. In more hyperpolarized conformations, it is thought that R2 or even R1 would occupy the charge transfer center (Tao et al. 2010). This model of gating charge motion provides a structural and mechanistic understanding for the existence of multiple closed states of the VSD, the existence of which was first proposed by Cole and Moore in 1960 to explain the observed delay in channel activation after prolonged strong hyperpolarizations (Cole & Moore 1960). The clear mechanistic importance of the pattern of charged residues within the transmembrane region of the VSD is demonstrated by the conservation of this pattern of charges throughout evolution (Yu & Catterall 2004; Bezanilla 2000).

The H\textsubscript{V} channel constitutes a unique member of the voltage-gated cation channel family. The remainder of this chapter will introduce the properties of this channel, its many important physiological roles and the scientific questions it poses.
1.3 **Voltage-Gated H⁺ Channels**

1.3.1 Discovery of Hᵥ channels

Voltage-gated H⁺ channels were first proposed to exist by Fogel and Hastings in 1972 for their model of bioluminescence in the single cellular eukaryotic algae *Noctiluca miliaris* (Fogel & Hastings 1972). However, the first biophysical characterization of voltage-gated H⁺ currents was not until ten years later when Thomas and Meech published their voltage-clamp studies on snail neurons (Thomas & Meech 1982). Thomas and Meech found that, upon depolarization, the membranes of these cells greatly increase their permeability to H⁺, which led them to propose that “H⁺ channels may be more widespread than hitherto suspected” (Thomas & Meech 1982). This proposal was confirmed by the subsequent discovery of Hᵥ channels in many different cell types from a diverse set of organisms (Barish & Baud 1984; DeCoursey 1991; DeCoursey & Cherny 1993; Kapus *et al.* 1993; Demaurex *et al.* 1993; Taylor *et al.* 2011; Smith *et al.* 2011).

The Clapham and Okamura groups published the first genes for Hᵥ channels in 2006. As explained in the previous section, the pattern of evolutionarily conserved charges within the VSD makes up a signature sequence. By using the sequences of known VSDs, Ramsey *et al.* and Sasaki *et al.* searched the human and mouse genomes with the aim of identifying any previously unknown VSD containing proteins (Ramsey *et al.* 2006; Sasaki *et al.* 2006). In 2006, both groups published the discovery of the previously unknown gene of the voltage-gated H⁺ (Hᵥ) channel (Ramsey *et al.* 2006; Sasaki *et al.* 2006).
1.3.2 Domain overview of Hᵥ channels—not your canonical voltage-gated channel

The sequence of hHᵥ1 is shown in Fig.1.5A and is very different from all previously described voltage-gated cation channels in that it does not have a 6-TM topology (see Fig 1.1). Hᵥ channels contain the first four transmembrane helices (S1-S4) of the VSD but lack the last two transmembrane helices that comprise the pore domain in the canonical 6TM channels (Kᵥ, Naᵥ and Caᵥ channels, Fig. 1.1). Nonetheless, when these genes are expressed in HEK cells, robust depolarization-dependent, Zn²⁺-sensitive, H⁺ currents can be measured (Ramsey et al. 2006; Sasaki et al. 2006).
Fig. 1.5 Primary structure and transmembrane topology of hHv1

(A) Amino acid sequence of hHv1. The predicted unstructured N-terminus is underlined in red, the predicted transmembrane helices are highlighted in grey and labeled S1-S4, the predicted C-terminal coiled-coil (Lupas et al. 1991) is underlined in black, the putative short helices within the N-terminus are boxed by blue dashed lines, the two known phosphorylation sites are boxed in green (Musset et al. 2010a) and the two histidines that are known to bind Zn$^{2+}$ are boxed in orange (Ramsey et al. 2006). Critical residue D112 in S1 is boxed in blue. (B) Transmembrane topology of the hHv1 channel. Horizontal black lines denote the approximate membrane boundaries (cytoplasm side down). The S4 gating charge residues are represented as “+” signs.
Human \( \text{H}_v \text{l} \) (h\( \text{H}_v \text{l} \)) is comprised of 273 amino acid residues with both the N- and C-termini residing in the cytoplasm (Fig. 1.5B). Secondary structure prediction identifies the four transmembrane helices of the VSD fold (highlighted in grey in Fig. 1.5A and labeled S1 through S4). Although it is very clear that these four transmembrane helices are present in the structure of the channel, the exact boundaries (i.e. the amino acid positions at which the helices start and terminate) are unknown. The first \( \sim 90 \) amino acid residues of h\( \text{H}_v \text{l} \) are predicted to be mostly disordered (Fig. 1.5A). The high proportion of negatively charged glutamate (E) and aspartate (D) residues, as well as the many proline (P) residues in the N-terminus, suggest an unstructured random coil. However, secondary structure prediction algorithms indicate that two short helices may be present in the N-terminus: one spanning residues A18-H27 (distant from the transmembrane helices in primary sequence) and the other spanning residues D87-S97, directly preceding S1 (Cole et al. 2008). Short amphipathic helices preceding the S1 have been observed in the structures of other voltage-sensor domains (VSDs) and have been termed S0 (Long et al. 2007; Butterwick & Mackinnon 2010). Therefore, it is possible that h\( \text{H}_v \text{l} \) also contains a S0 helix preceding S1. It is also interesting to note that a phosphorylation site that affects channel gating has been identified at T29, adjacent to the short predicted N-terminal helix. This suggests that this region of the protein may interact closely with the transmembrane domain (Musset et al. 2010a). The C-terminal end of human \( \text{H}_v \text{l} \) is predicted to comprise one half of a coiled-coil structure; a number of structural and biochemical studies examining this isolated sequence demonstrate that it forms a homodimer coiled-coil structure (Li et al. 2010; Fujiwara et al. 2012; Fujiwara et al. 2013a).
1.3.3 Biophysical features of H\textsubscript{v} channels

In addition to exhibiting near perfect selectivity for H\textsuperscript{+} (DeCoursey 2003b), the voltage-gated H\textsuperscript{+} currents measured in a variety of organisms share specific biophysical features, as described below.

\textit{ΔpH dependence of gating}

Very early on, it was found that the gating of the depolarization activated H\textsuperscript{+} current was not solely dependent on the transmembrane potential, but that it also strongly depended upon the pH difference across the membrane (ΔpH, defined as external pH [pH\textsubscript{o}] minus internal pH [pH\textsubscript{i}]; Byerly \textit{et al.} 1984). An example of currents elicited from whole-cell patch clamp recordings of HEK cells expressing hH\textsubscript{v.1} is shown in Fig. 1.6 at three different values of pH\textsubscript{o} (Fig. 1.6A pH\textsubscript{o} = 7.0, Fig. 1.6B pH\textsubscript{o} = 6.5 and Fig. 1.6C pH\textsubscript{o} = 6.0). It is clear from the current recordings that the different pH\textsubscript{o}s have an effect on the rate of channel opening and closing (Fig. 1.6A-C). By plotting an IV curve from the normalized tail currents, it becomes clear that the different pH\textsubscript{o}s also have a strong effect on the voltage dependence of the gating (Fig. 1.6D). Reducing pH\textsubscript{o} (increasing the external concentration of H\textsuperscript{+}) results in a significant shift of the threshold voltage (V\textsubscript{thr}, defined as the voltage at which you begin to see H\textsuperscript{+} current) to more depolarized potentials (rightward shift on the IV plot; Fig. 1.6D). This ΔpH dependence is also seen upon changes of pH\textsubscript{i} and results in the channels only ever opening at membrane potentials more positive than the Nernst equilibrium potential for H\textsuperscript{+} (E\textsubscript{H\textsuperscript{+}}; Fig. 1.6D; Cherny \textit{et al.} 1995; DeCoursey 2003b).
Fig. 1.6 Whole cell patch clamp recording of HEK cells expressing hHV1 channels
Current recordings from the same HEK cell expressing hHV1 channels at different pH_o (A) pH_o = 7.0, (B) pH_o = 6.5 and (C) pH_o = 6.0; pH_i = 6.5 for each recording. Voltage step protocols are represented schematically above each recording. (D) Normalized IV curves plotted from tail-currents. The arrows at the bottom indicate the different values of E_H for each ΔpH shown (calculated by equation 1.1).
Through their dependence on ΔpH, these channels become outward rectifiers: they only ever open in resting mammalian cells such that the electrochemical potential for H⁺ conduction is outward. In other words, Hᵥ channels are acid extruders, only opening to allow H⁺ to leave the cytoplasm. This property defines the many physiological roles played by the Hᵥ channels (discussed in the next section). Although models have been proposed for how this gating behavior could be achieved (Cherny et al. 1995; DeCoursey 2003b), the mechanism remains unknown and the amino acid residues involved in pH sensing on both sides of the membrane remain to be identified.

*Inhibition by Zn²⁺*

To varying degrees, all Hᵥ channels thus far characterized are inhibited by polyvalent metal cations, with Zn²⁺ in general being the most potent inhibitor (Byerly et al. 1984; Mahaut-Smith 1989; DeCoursey 2003b). It is thought that the major mechanism of inhibition is through the direct binding of Zn²⁺ to an extracellular receptor site on the channel, which stabilizes the hyperpolarized (closed) conformation and thereby shifts the voltage-dependence of gating to more depolarized potentials (Cherny & DeCoursey 1999; DeCoursey 2003b). The binding of Zn²⁺ was found to be highly sensitive to pHₒ and detailed competition studies indicated that H⁺ and Zn²⁺ compete for binding to the same site composed of multiple groups (Cherny & DeCoursey 1999). Furthermore, since both external H⁺ or Zn²⁺ binding stabilize the closed conformation, it was proposed that the groups responsible for Zn²⁺ binding may also be responsible in part for the ΔpH dependence of gating (Cherny & DeCoursey 1999).
After the discovery of the Hv gene, Ramsey et al. identified the Zn\(^{2+}\) binding site in hHv1 (Ramsey et al. 2006). The binding site bridges two histidine residues, one at position 140 (near the extracellular end of S2) and the other at position 193 (within the voltage-sensor paddle motif; see Fig. 1.5A). When either or both of these two histidines are mutated to alanine, Zn\(^{2+}\) inhibition is alleviated (Ramsey et al. 2006). However, a systematic study of the role of these histidine residues on the \(\Delta pH\) dependence of gating has yet to be published. It is also interesting to note that the Hv channel found in calcifying coccolithophores (see below) does not have histidine residues at these positions and yet remains inhibited by extracellular Zn\(^{2+}\) ions, indicating that an alternative receptor exists for Zn\(^{2+}\) in these channels (Taylor et al. 2011).

Conduction via protein mediated hydrogen-bonded chain

The conduction of H\(^{+}\) both in water and through proteins is dissimilar to that of other ions in that it does not occur via simple diffusion but via the formation of transient covalent bonds (de Grotthuss 1806; Cukierman 2006; DeCoursey 2003b). Channel-mediated conduction of H\(^{+}\) across the membrane has commonly been found to occur by two distinct mechanisms: as H\(_3\)O\(^{+}\), via a continuous water wire as in the gramicidin channel (Cukierman 2000), or as H\(^{+}\) via a hydrogen-bonded chain (the transfer of the H\(^{+}\) to a titratable amino acid side chain in the channel which then passes it to water on the other side of the membrane) as in the influenza M2 channel (Pinto et al. 1992; DeCoursey 2003b).

Which mechanism do Hv channels utilize for H\(^{+}\) conduction? By measuring the effect of buffer concentration, temperature and the deuterium (\(^{2}\)H) isotope on proton
conduction, DeCoursey and Cherny established that the rate of H\(^+\) conduction through H\(_v\) channels is not diffusion-limited (DeCoursey & Cherny 1996; DeCoursey & Cherny 1998; DeCoursey & Cherny 1997). This indicates that there is a rate-limiting step for H\(^+\) conduction as it permeates the channel. When comparing the temperature and deuterium isotope effects to other channels with established conduction mechanisms, such as the gramicidin and influenza M2 channels, the values found for hH\(_v\)1 are more consistent with a hydrogen-bonded chain mechanism, as opposed to a continuous water-wire mechanism (DeCoursey 2003b). This titratable residue was later identified in hH\(_v\)1 to be D112 on the S1 helix (Fig. 1.5A; Hille 2001; Musset et al. 2011). Mutation of this residue to valine results in a defective H\(^+\) channel unable to conduct H\(^+\); mutation to other amino acid residues results in Cl\(^-\) leakage through the channel (see Chapter 3 for a more complete discussion; Musset et al. 2011).

1.3.4 H\(_v\) channels diverse physiological roles

Although the first description of H\(_v\) channel currents was from snail neurons, the role that the channels play in neuronal physiology remains poorly understood. In humans, H\(_v\) channels are mainly found in cells of the immune system and in sperm cells (Barrett et al. 1982; DeCoursey 2010; Capasso et al. 2010; Lishko et al. 2010). In addition, a potentially significant role of H\(_v\) channel activity is being uncovered in cancer cells (Wang et al. 2012; Wang et al. 2013a). In this section I will briefly discuss a selection of the physiological roles that H\(_v\) channels play in humans, as well as some of the important roles they play in single-celled eukaryotes.
**Phagocytes**

H$_v$ channels have been best characterized physiologically for the role they play in stabilizing the activity of the NADPH oxidase during oxidative bursts in phagocytes’ phagosomes (DeCoursey 2010). Upon encountering a bacterium, a phagocyte engulfs it into a specialized vacuole known as the phagosome. Through the activity of the NADPH oxidase (which transfers electrons from NADPH to molecular oxygen, generating NADP$^+$ and H$^+$ in the cytoplasm and the superoxide ion [O$_2^-$] in the phagosome), the macrophage attacks the bacterium with reactive oxygen species (ROS; DeCoursey 2010). In this way, the activity of the NADPH oxidase results in the depolarization of the phagosome membrane (inside negative with respect to the cytoplasm) and the acidification of the cytoplasm. Because of the voltage-dependence of NADPH oxidase activity, this activity would be self-inhibitory in the absence of any compensatory mechanism (DeCoursey *et al.* 2003). H$_v$ channels in the phagosome membrane provide the required compensatory mechanism: they open in response to the NADPH-oxidase-induced membrane depolarization and conduct H$^+$ into the phagosome, simultaneously repolarizing the membrane and deacidifying the cytoplasm (DeCoursey *et al.* 2003; DeCoursey 2003a).

**Human sperm cells**

The H$_v$ channel is integral for regulation of the internal pH of the sperm cell (Lishko *et al.* 2010; Lishko & Kirichok 2010). The pH of the sperm cytoplasm is a major regulator of sperm cell motility, capacitation, hyperactivation and the acrosome reaction, all essential processes for the fertilization of the egg (Lishko *et al.* 2012). For example, the
capacitation process (by which sperm cells go from a quiescent state to an actively swimming state) was known to involve the extrusion of protons out of the sperm cytoplasm (Giroux-Widemann & Jouannet 1991; Hamamah & Gatti 1998). In 2010, Lishko et al. showed this proton efflux in human sperm cells is facilitated by Hᵥ channels (Lishko et al. 2010).

Upon the identification of role played by Hᵥ channels in sperm cell physiology, a potential mechanism for the role of Zn²⁺ in sperm cell activation was proposed (Lishko et al. 2010). Since the very early characterization of Hᵥ current, it was shown that Zn²⁺ is a potent inhibitor of the channel (see above; Mahaut-Smith 1989). It is telling that the highest concentration of Zn²⁺ in the human body is found in the seminal fluid (Saaranen et al. 1987; Lishko et al. 2012). Through inhibition of Hᵥ channels, a high concentration of Zn²⁺ would prevent premature sperm capacitation; conversely, removal of Zn²⁺ would promote proton efflux from the sperm cytoplasm. It has been demonstrated in rats that, upon entering the female reproductive tract, the Zn²⁺ from the seminal fluid is rapidly diluted (Gunn & Gould 1958). There is also evidence that the lipid-derived hormone anandamide present in the human reproductive system (Schuel & Burkman 2005) may have a direct activating influence on Hᵥ channels (Lishko et al. 2010).

Cancer
The metabolism of cancer cells is markedly different than that of normal cells (for a review see Griffin & Shockcor 2004). Cancerous cells require a large amount of energy to fuel their expansive growth. Therefore, many cancers are characterized by increased levels of metabolism, specifically increased activity of the glycolytic pathway (Gatenby
Furthermore, because of the high cell density of tumors, cancer cells tend to be hypoxic (Knowles & Harris 2001). Therefore, due to their high metabolism and their hypoxic environment, cancer cells start to accumulate high levels of H⁺ (a byproduct of anaerobic glycolysis) in their cytoplasm (Gatenby & Gillies 2007). Without a mechanism for extruding protons out of the cytoplasm, these cells would not be able to continue proliferating or to metastasize.

The discovery of the expression of hHv1 in highly metastatic breast and colorectal cancer cells, both from human cancer biopsies and established cancer cell lines, has led to the hypothesis that these channels are responsible for the regulation of cytoplasmic pH that allows the high proliferation of these cells (Wang et al. 2011; Wang et al. 2012; Wang et al. 2013a). Additionally, it has been demonstrated that Hv channels are overexpressed in the highly metastatic glioma cell SHG-44 and that inhibition of Hv channel activity by Zn²⁺ when these tumors are implanted into nude mice results in significantly smaller tumor size (Wang et al. 2013b). These findings indicate that Hv channels might be effective targets for anticancer drugs.

Calcification in Coccolithophores

Coccolithophores comprise a large group of phytoplankton that is found in all oceans around the globe (Winter & Siesser 1994). These unicellular algae generate elaborate calcium carbonate scales (coccoliths) of unknown physiological function (Marsh 1999). Nonetheless, because of the tendency of coccolithophores to grow into immense blooms, they are the most numerous calcifying organisms in the ocean. Coccolithophores significantly impact marine biogeochemical cycling and atmospheric chemistry by
incorporating inorganic carbon into their coccoliths and carrying it to the bottom of the ocean when they die (Rost & Riebesell 2004).

The process of coccolith formation occurs within an intracellular compartment and involves the combination of Ca$^{2+}$ and bicarbonate ($\text{HCO}_3^-$) to produce calcite ($\text{CaCO}_3$), releasing one mole of H$^+$ for every mole of CaCO$_3$ produced (Marsh 1999; Brownlee & Taylor 2004; Paasche 2001). Without a mechanism for H$^+$ removal from the cytoplasm, it is predicted that coccolith production would acidify the cell at a rate of $\sim$0.3 pH min$^{-1}$ (Taylor et al. 2011). The mechanism of H$^+$ dumping from the coccolithophores was not understood until the discovery that these cells express H$_v$ channels (Taylor et al. 2011). The activity of these H$_v$ channels allows for the maintenance of calcification by regulating cytoplasmic pH homeostasis, thereby influencing the composition of the atmosphere on a global scale.

**Bioluminescence in dinoflagellates**

In 1972, Fogel and Hastings first proposed the existence of voltage-gated proton channels as a component of their theoretical model describing the process of bioluminescence in dinoflagellates (Fogel & Hastings 1972). It was not until 2011 that the existence of H$_v$ channels in dinoflagellates was experimentally confirmed (Smith et al. 2011). The H$_v$ channels found in these single cellular algae are unique in that they activate at membrane potentials negative to the Nernst equilibrium potential for H$^+$, thus allowing for inward H$^+$ current (Smith et al. 2011). This feature is necessary for this H$_v$ channel to fulfill its proposed role in the triggering of bioluminescence by rapid H$^+$ influx into the specialized small organelles known as scintillons (Fogel & Hastings 1972). Additionally, this
distinctive gating behavior allows these H$_v$ channels to participate in the production of action potentials that carry the H$^+$ signals along the algal membranes to the scintillon organelles (Smith et al. 2011).

1.4 MOTIVATION AND AIMS OF THESIS RESEARCH

1.4.1 Many fundamental questions to tackle

H$_v$’s significant deviations from the canonical voltage-gated domain structure, namely, its lack of separate voltage-sensing and pore domains, raised a number of fundamental questions as to its mechanism of gating, conduction and regulation. How does the channel conduct H$^+$ without a pore domain? Is the gene identified actually the H$^+$ channel, or does the protein associate with some unknown factor in the HEK cell membrane to mediate H$^+$ conduction? Given that the pore helices create the multimerization interface in the K$_v$ channels, what is the multimeric state of the H$_v$ channel?

I joined the MacKinnon laboratory in 2008, less than two years after the first discovery of the H$_v$ genes by Ramsey and Sasaki (Ramsey et al. 2006; Sasaki et al. 2006). At the time, all that was known about the H$_v$ channel protein was its sequence and the histidine residues that were responsible for Zn$^{2+}$ binding (Ramsey et al. 2006); many of the biophysical and physiological studies mentioned in the previous sections had yet to be performed. In this thesis, I describe my efforts on the investigation of the molecular mechanisms of H$_v$ channel gating and conduction through attempts at functional and structural characterization.
In Chapter 2, I describe my work on the multimeric state of the hH\textsubscript{v}1 channel in membranes. Together with the postdoctoral fellow Dr Seok-Young Lee, I demonstrated that H\textsubscript{v} channels form dimers in the membrane and we identified the dimerization interface to be along the S1 helix in the membrane and along the coiled-coil in the cytoplasm (Lee \textit{et al.} 2008).

In Chapter 3, I describe our approach of studying the purified H\textsubscript{v} channels in detergent and reconstituted into vesicles. Whereas all other research on H\textsubscript{v} channels has been performed in cells, we decided to biochemically characterize the purified channel, taking advantage of the expertise in membrane protein expression and purification in the MacKinnon laboratory. By studying the purified protein reconstituted in lipid vesicles, we demonstrated for the first time that the hH\textsubscript{v}1 gene alone is able to conduct H\textsuperscript{+} (Lee \textit{et al.} 2009b). Furthermore, to identify amino acid residues that play an important role in H\textsuperscript{+} conduction, we purified and reconstituted mutated hH\textsubscript{v}1 channels and subjected them to functional analysis. We identified D112 as a candidate residue involved in H\textsuperscript{+} conduction and showed that the mutation of an equivalent position to aspartate in the isolated VSD of the prokaryotic K\textsubscript{v} channel K\textsubscript{v}AP increases the rate at which this protein conducts H\textsuperscript{+}.

In Chapters 4 and 5, I describe my efforts towards the structural characterization of H\textsubscript{v} channels. Determining the atomic resolution structure of a protein and using it to generate experimentally testable hypotheses is one of the most powerful ways to produce mechanistic understanding. Needless to say, this would be particularly useful for many of the poorly understood H\textsubscript{v}-channel biophysical properties. Encouraged by our development of expression and purification methods for hH\textsubscript{v}1 as well as by our colleague’s determination of the K\textsubscript{v}AP isolated VSD structure (Jiang \textit{et al.} 2003a), we
decided to pursue the structure of hH\textsubscript{V}1 by x-ray crystallography. Our efforts to this end are discussed in detail in Chapter 4.

In addition to the crystallographic approach, together with postdoctoral fellow Dr Joel Butterwick, I also examined H\textsubscript{V} channels in detergent by solution state NMR. Because of its sensitivity to the protonation state of amino acid residue side chains, NMR constitutes an ideal methodology for probing the conduction mechanism of H\textsubscript{V} channels. Additionally, NMR can be used to calculate protein structures and since Dr Butterwick had solved the structure of K\textsubscript{V}AP isolated VSD by this method (Butterwick & Mackinnon 2010), we thought this would be a complementary approach to solving the hH\textsubscript{V}1 structure. Our efforts on the NMR of H\textsubscript{V} channels are discussed in detail in Chapter 5.

Although the attempts at complete structural characterization of H\textsubscript{V} both by crystallography and NMR have thus far been unsuccessful, we were able to define the secondary structure of the channel for the first time and determine that the channel requires a lipid bilayer for the stabilization of its three-dimensional fold. Ongoing and future research into H\textsubscript{V} channel structure must focus on membrane like environments. For crystallography this includes detergent-lipid mixtures, bicelles and lipidic cubic phase crystallography. For NMR solution state studies can be performed on the channel in lipid nanodisks or solid state NMR can be attempted on pellets of reconstituted channels.

Significant progress has been achieved in the last 7 years since the H\textsubscript{V} genes were identified. However, many fundamental questions still remain. Complete mechanistic understanding of H\textsubscript{V} conductance and gating will ultimately require the atomic description of the protein in multiple states. As such, structural characterization of H\textsubscript{V} remains a pressing goal for the voltage-gated ion-channel field. Understanding of this
unique voltage-gated channel will contribute to our understanding of the diverse mechanisms that have evolved which allow voltage-gated cation channels to fulfill their diverse and important physiological roles.
CHAPTER 2: H\textsubscript{V} IS A DIMER IN THE MEMBRANE

Voltage-gated six-transmembrane (6-TM) cation channels (Na\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+}) contain voltage-sensor and pore domains (Hille 2001). In the case of voltage-gated K\textsuperscript{+} (K\textsubscript{v}) channels, four 6-TM subunits come together in the membrane to form a four-fold symmetric tetramer (Mackinnon 1991; Long \textit{et al.} 2007). The last two transmembrane helices (S5 and S6) from each subunit come together to form the pore domain, with the ion conduction pathway through the central four-fold access of symmetry (Yellen \textit{et al.} 1991; Doyle \textit{et al.} 1998). The first four transmembrane helices (S1-S4) form the voltage-sensor domain (VSD); the four VSDs of the tetramer arrange themselves around the periphery of the pore (see Fig. 1.3B on page 15; Long \textit{et al.} 2007). The multimeric interface of the K\textsubscript{v} channel is completely composed of the pore domain (S5 and S6) without any contribution from the VSDs (Long \textit{et al.} 2007).

Until the cloning of voltage-gated proton (H\textsubscript{v}) channels, it was thought that all voltage-gated cation channels had a 6-TM architecture, containing distinct VSDs and pore domain (Yu & Catterall 2004). However, the work of Ramsey \textit{et al.} and Sasaki \textit{et al.} revealed that the voltage-sensor domain can also exist independently, as the cloned sequences showed that H\textsubscript{v} channels contain only a voltage-sensor domain in the
membrane without a separate pore domain (Ramsey et al. 2006; Sasaki et al. 2006). Because the pore domain of other voltage-gated cation channels is instrumental in generating higher-order structure in the membrane (e.g. the tetramerization of the Kv channels), the oligomeric state of the Hv channel was unknown. This fundamental property of the channel can have significant repercussions for our mechanistic understanding of the channel’s gating and conduction. It has been shown that in order for VSDs to function properly in Kv channels two interfaces are necessary between the VSD and the pore (Lee et al. 2009a). Do Hv channels require support from other protomers in the membrane in order to properly function? The conduction pathway for other voltage-gated cation channels is found at an assembly interface (Yellen et al. 1991; Long et al. 2007). Where is the conduction pathway of Hv channels? Does it form at an oligomeric interface or do H+ conduct through the VSD itself?

In this chapter I will discuss my work carried out together with Dr Seok-Yong Lee, which addressed these questions through evaluation of the subunit stoichiometry of the human Hv1 (hHv1) channel. By site-specific cross-linking in cell membranes we were able to demonstrate that hHv1 is a dimer in the membrane and we were able to define the dimeric interface (Lee et al. 2008).
2.1 Human Hv1 Is a Dimer in the Membrane

2.1.1 Non-specific cross-linking in HEK cells

To probe the oligomeric state of hHv1, cell membranes isolated from HEK cells transfected with hHv1 cDNAs were subjected to the amino-group specific bifunctional cross-linker disuccinimidyl suberate (DSS) and visualized by western blot analysis using antibodies directed against the hHv1 channel coiled-coil. Amino-group specific cross-linkers have been successful in defining the oligomeric status of several membrane proteins (Albright et al. 2007; Aller et al. 2004). Recombinant Hv1 makes functional channels in HEK cells (Ramsey et al. 2006; Sasaki et al. 2006; Musset et al. 2008). hHv1 migrated at approximately 35 kDa in SDS-PAGE under reducing conditions, which is consistent with the molecular weight of a monomer (32 kDa; Fig. 2.1A). As the concentration of the cross-linker (DSS) was increased, a band migrating near 73 kDa appeared with a concomitant decrease in the monomer band. Further increase of cross-linker concentration yielded a series of weak bands corresponding to higher oligomers in addition to the strong dimer band. These were probably due to non-specific cross-linking with other membrane proteins or cross-linking between the hHv1 dimers. However, the appearance of strong dimer bands at low concentrations of DSS suggested that hHv1 forms a homo-dimer in the membrane. Dimerization in hHv1 is not a consequence of disulfide bond formation because mutants lacking cysteines showed the same DSS-mediated cross-linking pattern as wild type (Fig. 2.1B).
Fig. 2.1 hH\_1 is a dimer

Western blots of membranes from tsA201 cells transfected with (A) wild type and (B) cysless hH\_1 cDNAs and exposed to increasing concentrations of the amino-group specific cross-linker DSS (from left to right: No DSS, 25 \(\mu\)M, 75 \(\mu\)M, 250 \(\mu\)M, 2.5 mM). Membrane samples were run on SDS-PAGE under reducing conditions as per methods. All the gels that were used are 12%. Molecular weight markers are shown on the left side of each blot.

2.2 PROBING THE DIMERIC INTERFACE

2.2.1 Design of site-specific cross-links

The finding of a dimeric subunit stoichiometry for hH\_1 leads to the question of how two voltage sensors arrange relative to each other in the membrane. If the hH\_1 homo-dimer contains a two-fold rotation axis normal to the membrane, a single cysteine mutation close to the dyad axis should form a cross-linked dimer under oxidizing conditions, or in the presence of a bifunctional cross-linker. By sequence analysis, hH\_1 can be dissected
into three subdomains: an N-terminal acid- and proline-rich region, a transmembrane voltage-sensor domain, and a C-terminal coiled coil domain (see Fig. 1.5 on page 22 and Fig. 2.2). Using the voltage sensor of the Kv1.2-Kv2.1 paddle chimera structure as a reference (Long et al. 2007), we examined both the two naturally existing cysteine residues and a series of substituted cysteines on a natural-cysteine-free background (Fig. 2.2). Positions that were mutated to cysteine included: R100 (preceding S1), C107 and L120 (S1), I127 and N132 (S1-S2 loop), T145 and I155 (S2), E164 (S2-S3 turn), L173 and V187 (S3), Q194 (S3-S4 turn), L203 and I212 (S4), T222 (after S4), and C249 (putative coiled coil; Fig. 2.2). Limited proteolysis and preliminary biochemical data suggested that the N-terminal region is largely unstructured and does not affect the oligomeric state of the protein. Therefore, although the full-length hKv1 was used, the N-terminal region was not investigated in this cross-linking study.
Fig. 2.2 Introduction of cysteine mutations into hH\textsubscript{V}1

(A) The positions that were mutated to cysteine are indicated by filled circles on the voltage-sensor structure of the K\textsubscript{V}1.2-2.1 paddle chimera (PDB ID: 2R9R). The S1-S2 loop from the paddle chimera was drawn as a shorter loop to match the length of hH\textsubscript{V}1.

(B) The amino acid sequence of hH\textsubscript{V}1. Predicted transmembrane regions, based on the structure of the paddle chimera and hydropathy, are highlighted gray. Residues mutated to cysteine are colored red. C107 and C249 are natural cysteines. The region corresponding to the coiled coil was calculated by the program COIL (Lupas \textit{et al.} 1991).
2.2.2 Dimer interface of H\textsubscript{v} channels

Each of the 14 cysteine-mutant cDNAs was transfected into tsA201 cells and membranes isolated from these cells were subjected to cross-linking reactions. The oligomeric state of each mutant was assessed by western blot analysis. Among the 15 mutants that we tested for cross-linking, only R100C failed to be expressed, by western blot analysis. As shown in Fig. 2.3A, all mutants migrated as a monomer under reducing conditions on SDS-PAGE. However, several mutants migrated as a dimer under non-reducing conditions (Fig. 2.3B). I127C, which is located in the loop between S1 and S2, formed an almost complete spontaneous disulfide bond, whereas C249—a natural cysteine located in the predicted coiled-coil domain—formed a significant quantity of spontaneous disulfide bond. N132C and T222C also showed some degree of disulfide bond formation. This pattern of disulfide bond formation suggests that S1 and the adjacent loop form a dimer interface on the extracellular side of the cell membrane and that the C-terminus, through a coiled-coil, forms a dimer interface on the intracellular side of the membrane.

Oligomer formation was also studied under conditions that ‘force’ cross-linking, either by the addition of the oxidant CuSO\textsubscript{4} and o-phenanthroline (CuP) or by the addition of the bifunctional cross-linker 1,3-propanediyl bismethanethiosulfonate (M3M), which has a 13 Å spacer separating the two cysteine-reactive methanethiosulfonate functional groups (Fig. 2.3C and D). Both reagents drove cross-links at I127C and at C249 nearer to completion. They also produced cross-linked cysteines to a lesser extent at positions near I127 (position 120 within S1 and position 132 in the S1-S2 loop) and near 249 (position 222 within the C-terminus).
Fig. 2.3 Dimer interface of hHv1

Western blots of membranes from tsA201 cells transfected with 14 individual hHv1 cysteine-containing mutant cDNAs that were subjected to (A) reducing reagent (5% (v/v) b-mercaptoethanol), (B) air oxidation, (C) CuP-induced cross-linking, or (D) M3M-mediated cross-linking as per methods. Cross-linked dimers migrate at approximately 73 kDa while monomers migrate at approximately 35 kDa. Other weak bands probably correspond to proteolytic fragments of hHv1. A mutant hHv1 in which the native cysteines were removed was included as a control (cysless). The number represents the amino acid position of the cysteine residue.
Forcing conditions also resulted in weak cross-links in two new regions: the S2-S3 turn—also known as the membrane interface anchor (positions 164 and 173) (Lee et al. 2005)—and the tip of the voltage sensor paddle (positions 187 and 194; Fig. 2.3C and D; Jiang et al. 2003a; Jiang et al. 2003b).

By covalently trapping rare conformations or molecular arrangements, forcing conditions can sometimes result in cross-link formation between residues that are only rarely in close proximity to each other. An example of this would be intermolecular cross-links (in this case inter-dimer cross-bridge formation between pairs of dimers). The blot in Figure 2.4A provides evidence for such dimer pairing by cross-linking of cysteines at position 173. When combined with a cysteine at 127 (which by itself formed a cross-linked dimer to near completion; Fig. 2.3B), cysteine at 173 resulted in the appearance of bands near the size of a tetramer (Fig. 2.4A). This observation is explicable if cross-links between position 173 cysteines formed between S2-S3 loops from separate dimers, because cross-links within a dimer would not produce a tetramer. A similar analysis of cysteine at position 194 in the background of a cysteine at 127 produced a different, yet equally interesting result. Cysteine at 194 did not produce tetramers in this setting, but reduced the degree to which cysteine at 127 formed a cross-linked dimer (compare Fig. 2.3C and D with Fig. 2.4A). The location of position 194 at the tip of the voltage sensor paddle, which is adjacent to the S1-S2 loop within a single voltage sensor of the dimer (see Fig. 2.2A), offers a possible explanation for this cross-linking outcome. By forming an intramolecular cross-link with cysteine 127 within the same subunit, cysteine at 194 would compete with cysteine 127-mediated dimer cross-linking. These more subtle aspects of the blots suggest that the S2-S3 turn and the voltage sensor paddle
are not actually part of the tight dimer interface, but that less specific cross-links are formed under forcing conditions.

A more extensive inspection of covalent dimer formation via cysteine residues within the S1 and S1-S2 loop showed differential propensities to react (Fig. 2.4B and C). Even among amino acids that we expect to reside outside the membrane (positions 124-128), positions 126 and 127 reacted to a greater extent, as if they were most optimally positioned to form a disulfide bridge relative to their molecular symmetry equivalents. Position 120, which we expect is buried in the membrane, formed a disulfide partially in the presence of CuP. Disulfide formation in a low dielectric environment suggests that position 120 is probably very near its molecular symmetry partner (Schwem & Fillingame 2006; Yu & Oprian 1999).
Fig. 2.4 Further study of the dimer interface.

(A) Distinguishing intra- from inter-dimer cross-linking. Numbers at the bottom of each gel indicate the cysteine position. (B) Specificity of cross-linking in the S1-S1 dimer interface. Transmembrane region of S1 (117 to 120) and the adjacent loop (124 to 128) were subjected to (B) air oxidation and (C) CuP - mediated oxidation.
2.3 **Discussion of Results and Subsequent Literature**

2.3.1 Schematic representation of the hH\(_V\)1 dimer

These cross-linking data support the conclusion that H\(_V\)1 exists in the cell membrane as a dimer of identical voltage sensor protomers. Two regions of contact between the voltage sensors correspond to the extracellular C-terminal side of S1 and the adjoining S1-S2 loop and intracellular C-terminus, which may form a coiled coil structure. The native cysteine at position 249 may help to stabilize the dimer at the coiled coil but a disulfide bridge at this position is not essential for holding the dimer together. We conclude that two voltage sensor protomers of a dimer come into direct contact at both the extracellular and intracellular side of the membrane. A schematic representation of the H\(_V\)1 dimer, based on the known crystal structures of K\(_V\) channel voltage sensors and the contact surfaces identified in this study, is shown in Fig. 2.5 (Jiang *et al.* 2003a; Long *et al.* 2005; Long *et al.* 2007).
Fig. 2.5 A model of the hH\textsubscript{v}1 dimer

(A) Topology diagram showing the dimer arrangement of hH\textsubscript{v}1 transmembrane regions based on the cross-linking data, viewed from the extra-cellular side. An ellipsoid denotes the 2-fold rotation axis normal to the membrane. (B) A cartoon representation of the hH\textsubscript{v}1 dimer. Transmembrane helices are labeled. The helical representation of the putative interfacial region (after S4) and the predicted coiled coil region are also included.
Contemporary publications from two groups corroborated and complemented our discovery that \( \text{H}_V \) channels are dimers in the membrane (Koch et al. 2008; Tombola et al. 2008). By expressing two differentially tagged mouse \( \text{H}_V \) channels subunits in HEK cell and performing pull-down experiments, Koch et al. showed complex formation between the two \( \text{H}_V \) constructs (Koch et al. 2008). Furthermore, they demonstrated that the amount of detectable complex is significantly reduced by deletion of the C-terminal coiled coil, providing further evidence for the importance of the coiled-coil in stabilizing the dimer interface (Koch et al. 2008). Using single molecule photobleaching on GFP-tagged \( \text{hH}_V \)1, Tombola et al. also demonstrated that \( \text{H}_V \) channels are dimers in *Xenopus* oocyte membranes (Tombola et al. 2008). Our work, together with the work from these other two groups, established that \( \text{H}_V \) channels are dimers in the membrane.

### 2.3.2 Each subunit has a separate \( \text{H}^+ \) conduction pathway

Many membrane proteins exist as oligomers of identical or similar subunits. In many cases, the need for an oligomeric structure is clear: potassium channels, for example, require four subunits to form a single ion conduction pathway in between the subunits (Doyle et al. 1998). In other membrane proteins, the reason for an oligomeric structure is less clear: in aquaporin channels (Murata et al. 2000) and in CIC Cl\(^-\) channels (Dutzler et al. 2002), the water and ion conduction pathways are formed entirely by atoms of a single protomer and yet these transport proteins are tetramers and dimers of identical protomers, respectively.

Into which category does the \( \text{H}_V \) channel fall? Based on electrophysiological studies showing that mutant \( \text{K}_V \) channel voltage sensors can themselves conduct ions
(Tombola et al. 2005) or H⁺ (Starace & Bezanilla 2004) across the membrane, and based on atomic structural studies showing that Kᵥ channel voltage sensors contain protonatable chemical groups extending most of the way across the membrane (Long et al. 2007), it is not difficult to imagine that H⁺ flows through individual voltage sensors in Hᵥ channels. In other words, each sensor might contain its own H⁺ conduction pathway.

The contemporary work by Tombola et al. provided evidence for this claim (Tombola et al. 2008). By generating tandem hHᵥ1 dimers (i.e., both subunits expressed on a single polypeptide) with specific cysteine mutations in each subunit and reacting them with a cysteine-specific reagent that disrupted H⁺ conduction, they demonstrated that each subunit contributed approximately 50% of the total H⁺ current (Tombola et al. 2008). Moreover, by making a chimeric Hᵥ channel in which the N- and C-termini were replaced by those of the voltage-sensor phosphatase from Ciona intestinalis (Ci-VSP), Tombola et al. were able to monomerize the channel and demonstrate that the monomerized channel was still able to conduct H⁺ (Tombola et al. 2008).

More recently mutagenesis studies on hHᵥ1 by Musset et al. found that a single point mutation, D112V, is able to completely abolish H⁺ conduction (Musset et al. 2011). Further mutagenesis data on hHᵥ1 suggest that D112 interacts with the S4 gating-charge arginines, indicating that this side chain is directed into the central cavity of the VSD itself and not located at the dimeric interface (Berger & Isacoff 2011). This finding further supports the notion that each subunit contains a separate conduction pathway.
2.3.3 H₉ channel dimer and cooperativity

Sometimes, multiple subunits allow active sites to function in a non-independent manner. Hemoglobin is a well-known example in which an oligomeric structure underlies cooperativity (Perutz 1989), allowing a steep relationship between oxygen saturation and oxygen partial pressure. In ClC Cl⁻ channels, one form of gating might arise from its dimeric architecture (Bykova et al. 2006). Thus, oligomeric architectures in both soluble and membrane proteins can allow for much more than the multiplicity of active sites.

Electrophysiological studies have demonstrated strong cooperativity between the two subunits of the H₉ channel dimer (González et al. 2010; Tombola et al. 2010; Musset et al. 2010b). Using voltage-clamp fluorometry and specifically labeling Ciona intestinalis H₉ channels on S4 with a fluorescent probe, Qui et al. demonstrated that each subunit undergoes independent conformational changes prior to a concerted opening of the H⁺ conduction pathway (Qiu et al. 2013). It has also been noted that truncation of the channel on the N- and C-terminus (or the C-terminus alone) removes the cooperativity of H₉ channel gating, which has been argued to be due to monomerization of the channel (González et al. 2010; Tombola et al. 2010; Musset et al. 2010b).

Although it is clear from Koch et al.’s pull down experiments that truncating the termini of H₉ channels reduce the proportion of dimer observed, there is still some dimer present, indicating that the truncated channel subunits may still be associated in the membrane and may only come apart during the many washes of the pull-down protocol (Koch et al. 2008). Furthermore, Fujiwara et al. demonstrated that mutation of three amino acid residues in the C-terminal coiled coil is sufficient for disruption of the cooperative gating without disruption of the dimer (Fujiwara et al. 2012). Therefore, the
cooperativity of gating can be modulated independently of channels’ multimeric state in the membrane. Hence, simply because deletion of the C-terminus removes cooperative gating does not necessarily mean the channel has become monomeric. In fact, evidence is mounting that, by affecting the oxidation state of a native cysteine residue in the coiled coil, C249 in hH₄₁ (shown in Fig. 2.3B to be able to spontaneously form a inter-subunit disulfide bond), the cell is able to tune the cooperativity of the channel, thereby physiologically linking channel activity to the redox state of the cell (Fujiwara et al. 2012; Fujiwara et al. 2013b).

2.3.4 Mechanistic Implications of the dimer

In H₄₁ channels, a dimeric architecture might be related to the mechanics of channel gating. It is interesting to note that the location of the extracellular dimer interface in the H₄₁ channel corresponds well to the region of contact between voltage sensor and pore in Kᵥ channels (Long et al. 2007). In Kᵥ channels, the contact between S1 and the pore at the extracellular surface is proposed to serve as a mechanical fixed point so that motions of the voltage sensor paddle (S3 and S4) are efficiently transmitted to the pore’s gate (Long et al. 2007; Lee et al. 2009a). By analogy, contacts between S1 helices in the H₄₁ dimer might serve a similar function by fixing S1 and S2, thus permitting the voltage sensor to move and open a conduction pathway within each voltage sensor.

However, the work of Tombola et al. on the monomerized Hᵥ-channel chimera, in which both N- and C-termini are replaced by sequences from Ci-VSP, indicates that Hᵥ channels are able to function in the absence of the dimer (Tombola et al. 2008). Additionally, the voltage-sensor phosphatase (VSP) functions as a monomer in the
membrane, indicating that not all VSDs require a fixed support at the extracellular side of S1 to function (Kohout et al. 2008). If they are able to function as monomers, what makes the mechanism of H⁺ channels and VSP different from the 6-TM voltage-gated cation channels, which have been shown to require a fixed support at the extracellular side of S1 in order to function (Lee et al. 2009a)?

All VSDs are able to change conformation in response to changes in the transmembrane voltage. However, the VSDs of the 6-TM voltage-gated cation channels are electromechanical force transducers: the conformational changes need to be transmitted to the pore helix, via the S4-S5 linker and the S6 helix bundle, in order to open and close the ion conduction pathway (Long et al. 2007; Lee et al. 2009a). In order to efficiently transmit force to the pore domain, a fixed anchor in the membrane is required (Lee et al. 2009a). Are the VSDs of H⁺ channels and VSPs electromechanical force transducers? The conformational changes of these VSDs are not coupled to opening or closing of a separate pore as in the case of the canonical 6-TM voltage-gated cation channels. If the role of these non-canonical VSDs were simply to undergo conformational changes and not to transmit force, they would not require a fixed anchor in the membrane. Since the conduction pathway for H⁺ in H⁺ channels is located within the VSD itself, conformational changes of the VSD without force transduction should be sufficient to open the channel. Therefore, the purpose of the H⁺ dimer would not be to provide a fixed anchor to allow force transduction but to allow for modulation of gating through the regulation of cooperativity discussed in the previous section, and hence the channels would still be functional as monomers.
2.3.5 Implications of the dimer on future structural studies

In order to fully understand the mechanism and regulation of cooperativity in H\textsubscript{\text{v}} channels structures of both the reduced and oxidized dimers would be required (Fujiwara et al. 2013b). However, Tombola et al. show that a monomerized H\textsubscript{\text{v}}-VSP chimera is functional as a monomer (Tombola et al. 2008). Therefore, it should be possible to gain insight concerning the gating and conduction of H\textsubscript{\text{v}} channels from the structure of a monomerized channel. In order to confirm that the monomeric channel is functional, constructs that disrupt dimerization should be built in the absence of chimerization and functionally characterized. These channels will have to be shown to be monomeric in membranes by their inability to form site-specific cross-links along S1. The C-terminally truncated channels (discussed above) that lack cooperativity during gating have not been experimentally shown to be monomeric and therefore may still exist as dimers in the membrane. If the truncated channels are found to still be dimeric in the membrane it may be possible to disrupt the dimer by introduction of tryptophan amino acid residues along the S1 dimer interface. Due to its large bulky side chain tryptophan has been used successfully in other studies to disrupt membrane protein structure (Sharp et al. 1995).

Alternatively, it has been suggested that due to its lack of a C-terminal coiled-coil the H\textsubscript{\text{v}} channel found in the dinoflagellates Karlodinium veneficum may exist as a monomer (Smith et al. 2011). However, this has yet to be experimentally verified. As discussed above, a definitive demonstration that monomeric H\textsubscript{\text{v}} channels are functional would significantly contribute to our understanding of the mechanism of gating and greatly improve our confidence in interpretations of structural studies on monomerized channel in detergent (see Chapters 4 and 5).
CHAPTER 3: FUNCTIONAL RECONSTITUTION OF HUMAN Hv1

Due to the complexity of the proteic component of a cell’s cytoplasm and membrane, it can be very difficult to determine the function of each of the individual proteins. When Sasaki et al. and Ramsey et al. expressed the putative Hv channel proteins in HEK cells, they observed the robust generation of voltage-gated H⁺ currents (Ramsey et al. 2006; Sasaki et al. 2006). However, whether this H⁺ conduction occurred through the putative Hv gene product was unclear. Given that the Hv protein has homology to the regulatory voltage sensor domain (VSD) of canonical voltage-gated cation channels, but lacks the pore domain responsible for ion conduction, it remained possible that Hv was not an ion channel at all, but, instead, an accessory subunit for the modulation of a separate, unknown endogenous HEK cell membrane protein. In order to determine whether or not the putative Hv channel is in fact responsible for H⁺ conduction a reductionist approach was necessary; the activity of the putative channel had to be assessed in isolation from all other cellular proteins.

In this chapter, I will discuss my work in collaboration with Dr Seok-Yong Lee addressing this issue (Lee et al. 2009b). Working with the eukaryotic expression vector Pichia pastoris, we were able to develop a protocol for the purification and reconstitution of human Hv1 (hHv1) into lipid vesicles. By assaying the activity of the purified
reconstituted channel using a fluorescence-based concentrative uptake assay, we were the first to demonstrate that the putative \( H_v \) channel gene product alone is in fact responsible for \( H^+ \) conduction. By reconstituting the protein at different protein-to-lipid ratios, we were able to demonstrate that the majority of the \( hH_v1 \) channels that we purified were functional, validating our purification protocol for use in more detailed biochemical and structural studies. As controls, we reconstituted several other voltage-gated channels to prove that the \( H^+ \) flux observed was specific to \( H_v \) channels and not due to non-specific leaks caused by the presence of protein in the membranes. Extensive mutagenesis studies of purified and reconstituted \( hH_v1 \) identified D112 on S1 as an important amino acid residue for \( H^+ \) conduction. Furthermore, mutation of the equivalent position to aspartate on the \( K_v \) AP isolated VSD increased the rate of \( H^+ \) flux through this protein, indicating that this residue is sufficient to promote \( H^+ \) conduction through VSDs. A model of \( H^+ \) uptake qualitatively recapitulated our experimental results. These findings will be discussed in the context of the more recent \( H_v \) channel electrophysiological literature.
3.1 The Putative Hᵥ Channel Mediates H⁺ Flux in Vesicles

3.1.1 Expression, Purification and Reconstitution of Human Hᵥ1

Heterologous expression of hHᵥ1 channels was attempted in both prokaryotic (Escherichia coli) and eukaryotic vectors (Pichia pastoris yeast, Sf9/baculovirus insect cells and HEK mammalian cells). Although expression of extractable channel was low in each system, it was determined that Pichia pastoris provided sufficient protein for the reconstitution experiments. Due to the poor expression, the high affinity C-terminal 1D4-tag was needed to isolate the low-abundance channel protein from high-abundance endogenous contaminants. The channels were extracted from the yeast membranes using dodecylmaltoside (DDM) detergent and purified by affinity chromatography on an anti-1D4 antibody resin, followed by size exclusion chromatography (see Materials and Methods for details). Purified protein appeared as a single band corresponding to the expected size of a monomer on gel electrophoresis under denaturing and reducing conditions (Fig. 3.1A). Reconstitution of Hᵥ protein into lipid vesicles was carried out by detergent removal from channel-detergent-lipid mixtures via dialysis, using different protein to lipid ratios. As a control for tight lipid bilayer formation, reconstitution of empty vesicles was carried out in parallel in the absence of protein, allowing us to measure any non-specific membrane leakage.
Fig. 3.1 H⁺ flux into vesicles containing recombinant Hv channels

(A) SDS-PAGE gel showing purified Hv channels. Lane 1: molecular weight marker, 2: final wash, and 3: hHv-1D4 eluted with 0.4 mg/ml 1D4 peptide. (B) Fluorescence-based H⁺ flux assay. Vesicles (cyan) loaded with high concentration of K⁺ are diluted into low concentration K⁺ buffer containing the fluorescence dye ACMA (9-amino-6-chloro-methoxyacridine). Addition of valinomycin (red), a K⁺ selective ionophore, results in K⁺ efflux, which generates a driving force for H⁺ influx. If there is a H⁺ channel (blue) in the vesicle membrane, pH inside the vesicle will decrease. This pH decrease is monitored by ACMA because the protonated form, which becomes trapped inside vesicles, loses fluorescence whereas unprotonated ACMA diffuses freely across the membrane (Zhang & Forgac 1994). (C) Fluorescence-based H⁺ flux assay for vesicles with and without hHv1 colored blue and red, respectively (n = 5). Error bars indicate standard error of the mean. Valinomycin and CCCP are added at the indicated time points.
3.1.2 Monitoring $H_v$ channel conduction by fluorescence

To study $H^+$ flux into lipid vesicles containing human $H_v$ channels, we used the fluorescence-based concentrative uptake $H^+$ flux assay depicted in Fig. 3.1B. This assay was originally developed to study $H^+$ flux through V-ATPase (Zhang & Forgac 1994). Vesicles were reconstituted in the presence of 150 mM K$^+$ and diluted 20-fold into buffer containing 7.5 mM K$^+$ generating a 10-fold K$^+$ gradient across the membranes. Upon addition of the K$^+$ selective ionophore valinomycin, the K$^+$ diffuses out of the vesicles, generating an electric potential across the vesicular membrane of approximately -60 mV (negative inside relative to outside, calculated by equation 1.1). If a $H^+$ conduction pathway is present in the membrane, the negative electrical potential inside the vesicles will cause $H^+$ to enter, lowering the internal pH. We monitored the changes in pH by a $H^+$-induced quenching of the fluorophore 9-amino-6-chloro-2-methoxyacridine (ACMA).

Fig. 3.1C shows the fluorescence change caused by the addition of valinomycin to a sample of empty vesicles and to a sample of hH$_v$1 reconstituted vesicles at a protein to lipid ratio of 1:100 (wt:wt). The empty vesicles exhibited a very gradual fluorescence change, consistent with there being a very slow non-specific leak for $H^+$ entry until the proton ionophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) is added. Vesicles containing $H_v$ channels showed a robust change in fluorescence upon addition of valinomycin, consistent with the $H_v$ channel providing a specific pathway for $H^+$ entry. The further reduction in fluorescence brought about by the addition of CCCP reflects a small fraction of empty vesicles. The ~15% of empty vesicles seen even at high protein-to-lipid ratios stems from a population of vesicles that appear incapable of incorporating functional $H_v$ channels (see below). Similar fractions of reconstitution deficient vesicles
have been described previously in studies of other channels and transport proteins (Eytan 1982; Goldberg & Miller 1991; Heginbotham et al. 1998).

3.1.3 The Majority of Reconstituted \( \text{Hv} \) Channels are Functional

In order to further investigate the functioning of \( \text{Hv} \) channels, we performed the reconstitution at various protein-to-lipid ratios (Fig. 3.2A). The fluorescence-based assay, which indirectly measures \( \text{H}^+ \) flux through an unknown relationship between \( \text{H}^+ \) concentration and fluorescence, precludes quantitative determination of \( \text{H}^+ \) conduction rates (Fiolet et al. 1974). The assay does, however, allow us to estimate the fraction of total channels in the reconstitution that are functional, as explained below.

The fluorescence decay brought about by the addition of valinomycin (\( \text{F}_{\text{Hv}} \)) is proportional to the number of vesicles that contain at least one functional \( \text{Hv} \) channel. The fluorescence decay brought about by valinomycin plus CCCP (\( \text{F}_{\text{total}} \)) is proportional to the total number of vesicles. Given that we know the mass protein to mass lipid ratio in the reconstitution, and that the reconstitution occurs efficiently (Fig. 3.2B), with a few assumptions we can calculate the mean number of channels per vesicle \( \mu \) from equation 3.1, where \( g_{\text{Hv}} \) and \( g_{\text{L}} \) are the grams of \( \text{Hv} \) channel and lipid added, \( r \) is the estimated average radius of a vesicle, \( M_{\text{L}} \) is the molecular weight of the average lipid molecule (754 Da), \( \sigma \) is the estimated area per lipid molecule and \( M_{\text{Hv}} \) is the molecular mass of the \( \text{Hv} \) channel dimer (70,000 Da).

\[
\mu = \frac{8g_{\text{Hv}}\pi r^2 M_{\text{L}}}{g_{\text{L}}\sigma M_{\text{Hv}}} \quad \{3.1\} 
\]
Fig. 3.2 H\(^+\) flux into vesicles containing H\(_v\) channels at various protein-to-lipid ratios

(A) Fluorescence-based H\(^+\) flux assay for vesicles containing a decreasing number of H\(_v\) channels. Protein-to-lipid ratios of 1:100 (dark blue, n = 5), 1:500 (pink, n = 2), 1:1000 (orange, n = 3), 1:5000 (yellow, n = 3), 1:10,000 (cyan, n = 4), 1:20,000 (light green, n = 3), 1:40,000 (green, n = 4), 1:60,000 (violet, n = 3) and empty vesicles (red squares, n = 4) are plotted (error bars represent the standard error of the mean, range of mean for 1:500).

(B) Sucrose cushion of vesicles containing H\(_v\) channels. Numbers denote the fractions collected from top to bottom.

(C) Determination of the fraction of functional H\(_v\) channels. Plot of \(\mu\) versus the ratio of fluorescence decay contributed by H\(_v\) containing vesicles over the total fluorescence decay by addition of CCCP where \(\mu\) is the ratio of the number of channels over number of vesicles calculated with equation 3.1. The two curves are derived from equation 3.2 with \(\phi\) (fraction of functional H\(_v\)) = 1.0, \(\theta\) (fraction of reconstitution deficient vesicles) = 0.15 (red) and \(\phi = 0.5, \theta = 0.15\) (green).
The main assumptions are that the lipid head group area is 63 Å² (DeCoursey 2003b; Rand & Parsegian 1989) and that the vesicles are uniform in size with a radius of 100 nm (Moffat et al. 2008). If incorporation of Hv channels into vesicles is random, then we expect equation 3.2 to be true, where \( \theta \) is the fraction of reconstitution deficient vesicles (~15%, measured directly from the data) and \( \phi \) is the fraction of channels that are functional (Heginbotham et al. 1998).

\[
F_{Hv}/F_{total} = (1 - \theta) \left[ 1 - \exp\left(\frac{-\phi \mu}{(1 - \theta)}\right) \right]
\]  \hspace{1cm} \{3.2\}

The red curve in Fig. 3.2C corresponds to the curve generated from equation 3.2 with \( \phi = 1.0 \). To ensure that this conclusion is not mistakenly based on an incorrect assumption of the vesicle radius (which we have not measured but estimate from values in the literature), we fit the data using alternative values. At 70 nm, vesicles would be too numerous given the number of channels: \( \phi \) would have to be greater than 1.0, which is physically impossible. At 130 nm, the best fit still corresponds to a \( \phi \) value of approximately 0.75. Therefore, even given the degree of uncertainty introduced by our assumptions of vesicle radius, uniformity, and lipid molecule surface area, the data support the conclusion that the majority of Hv channels in the reconstitution are functional.
3.1.4 H⁺ Flux is Specific to Hᵥ Channels

The small conductance inferred from this study (see the section on modeling the flux below), raises the concern that the H⁺ flux into the vesicles may be due to a non-specific leak resulting from the presence of protein in the membranes. To examine this possibility, we expressed, purified and reconstituted three additional proteins at equivalent protein to lipid ratios (to match the number of VSDs per vesicle): full-length KᵥAP (KᵥAP), KᵥAP isolated VSD (KᵥAP VSD), and Kᵥ1.2-2.1 paddle chimera (paddle chimera). The fluorescence-based H⁺ flux analysis of these proteins showed that vesicles containing either KᵥAP or paddle chimera generated very little fluorescence decay (Fig. 3.3). Because both KᵥAP and paddle chimera contain K⁺ pores, the K⁺-driven membrane potential may be established before valinomycin addition. Initial fluorescence values with vesicles containing these channels were very similar to other vesicle preparations (Hᵥ or empty), suggesting that no H⁺ influx occurred before the first point of data collection. Interestingly, vesicles containing KᵥAP VSD exhibited fluorescence decay but at a significantly slower rate than vesicles containing Hᵥ channels (Fig. 3.3). Sucrose cushions of these vesicles confirmed efficient reconstitution of all proteins. These data suggest that the H⁺ flux through Hᵥ is not simply a manifestation of membrane protein reconstitution into the vesicles: rapid H⁺ flux is specific to Hᵥ. The slower H⁺ flux observed for KᵥAP VSD might reflect an intrinsic H⁺ conduction potential of the VSD, which is suppressed by its association with the K⁺ channel pore.
Fig. 3.3 Specific H⁺ permeation through hHv1

Fluorescence-based H⁺ flux assay for vesicles containing hHv1 (dark blue, n=5), KvAP VSD (green, n=4), KvAP (dark green, n=4), paddle chimera (cyan). Empty vesicles are shown in red. Error bars indicate standard error of the mean. Valinomycin and CCCP are added at the indicated time points. KvAP, KvAP VSD, and paddle chimera channels were expressed and purified according to published procedures (Jiang et al. 2003a; Long et al. 2007). Reconstitutions were carried out as described in materials and methods with the following protein to lipid ratios (wt:wt) 1:200 (KvAP VSD), 1:100 (KvAP), and 1:50 (paddle chimera).
3.2 Mutagenesis of the \( \text{H}_\nu \) Channel Transmembrane Domain

3.2.1 \( \text{H}^+ \) flux through mutant hH\(_\nu\)1 channels

In order to find the amino acid residues responsible for \( \text{H}^+ \) conduction through the transmembrane domain of \( \text{H}_\nu \) channels, we conducted extensive mutagenesis studies. Because hydrophilic residues are required for specific interaction with ions in the hydrophobic interior of the membrane, we focused our mutagenesis on polar and charged amino acids within the transmembrane domain. Fig. 3.4 shows the transmembrane sequence of hH\(_\nu\)1 aligned to the equivalent sequence of the K\(_\nu\)1.1 channel Shaker, highlighting the 14 positions that were individually mutated to alanine or leucine. Each of the mutant channels was expressed, purified and reconstituted into lipid vesicles at a protein to lipid ration of 1:100 (wt:wt). To ensure that the mutations did not significantly affect the reconstitution efficiency of the channel, we ran sucrose cushion flotation assays on each of the reconstituted mutant channels.

The activity of each mutant was tested by the fluorescence-based concentrative uptake flux assay described in Fig. 3.1B and compared to that of reconstituted wild type hH\(_\nu\)1 and empty vesicles. Mutation of a residue that is required for \( \text{H}^+ \) conduction should result in the absence of fluorescence quenching. The data from the reconstituted mutant channels presented in Fig. 3.5A-D are organized by the transmembrane helix (S1-S4) on which the mutated residue resides. However, the mutants can also be organized by their effect on \( \text{H}^+ \)-mediated fluorescence quenching and it is in this context that I will discuss them below.
Fig. 3.4 hH\textsubscript{v}1 transmembrane residues targeted for mutagenesis

The transmembrane sequence of hH\textsubscript{v}1 is shown aligned to the equivalent sequence of the K\textsubscript{v}1.1 channel Shaker. Numbering at the bottom corresponds to the hH\textsubscript{v}1 amino acid position. The putative transmembrane helices are highlighted in grey and labeled S1-S4. The sites of the mutations are highlighted in red with the exception of position D112 that is highlighted in green. The phenylalanine that corresponds to the phenyalanine gap in S2 is highlighted in cyan (see Fig. 1.4).
**Fig. 3.5** H⁺ flux into vesicles containing mutant hHv1 channels

Fluorescence-based H⁺ flux assay for vesicles containing mutant hHv1 channels compared to vesicles containing wild type channels and empty vesicles. (A) Mutations along S1. (B) Mutations along S2. (C) Mutations along S3. (D) Mutations along S4. In each case valinomycin was added after 150 s and CCCP was added after 450s except in the case of D112L were CCCP was added after 900s. For empty vesicles CCCP was added at 1350 s (A) and 780 s (B C and D). All channels were reconstituted at a protein-to-lipid ratio of 1:100 (wt:wt). Each curve corresponds to the average of 3-4 repetitions with the error bars indicating the standard error of the mean.
3.2.2 Mutations with small or no effect on H⁺ conduction

Many of the mutations investigated had little or no effect on the H⁺ conduction when compared to the wild type channel (Fig. 3.5A-D). These included E119L on S1 (Fig. 3.5A), all residues tested on S2, H140A, S143A, E153A and K157A (Fig. 3.5B), D185A and H193A on S3 (Fig. 3.5C), as well as N214A on S4 (Fig. 3.5D). Although many of these mutants showed some effect on either the initial rate of H⁺ conduction (e.g. D185A on S3; Fig. 3.5C) or on the fraction of empty vesicles measured (e.g. H140A on S2; Fig. 3.5B), none of these mutants completely abolished H⁺ conduction. Differences in the fraction of empty vesicles indicated a higher proportion of non-functional channels in the vesicles. However, in each case, a significant proportion of the channels remained functioning and we are able to clearly observe H⁺ conduction. Small effects of the mutations on the initial rate of fluorescence quenching may indicate a role for those amino acids in H⁺ conduction; however, those roles are not critical, given that conduction is only slightly affected.

One Hᵥ channel mutation that was reported to disrupt H⁺ conduction in oocyte membranes expressing hHᵥ1 channels is N214R (Tombola et al. 2008). However, when we expressed purified and reconstituted this mutant at different protein-to-lipid ratios and tested it in our flux assay, we did not observe any significant difference in H⁺ conduction compared to wild type hHᵥ1 channels (Fig. 3.6A). Another mutagenesis study on the mouse Hᵥ (mVSOP) channel expressed in HEK cells and characterized by whole-cell patch clamp electrophysiology also found that the equivalent mutation N210R results in functional channels (Sakata et al. 2010).
Fig. 3.6 H⁺ flux into vesicles containing additional mutants of the hH₁V₁ channel

Fluorescence-based H⁺ flux assay for vesicles containing mutant hH₁V₁ channels compared to vesicles containing wild type channels and empty vesicles. (A) Dilution series of the N214R mutation on S4. (B) Mutations at position D112 on S1; all channels were reconstituted at a protein-to-lipid ratio of 1:100 (wt:wt). In each case valinomycin was added at 150 s and CCCP was added where indicated. Each curve corresponds to the average of 3-4 repetitions with the error bars indicating the standard error of the mean. (C) Sucrose cushion of vesicles containing D112L hH₁V₁ channels demonstrating efficient reconstitution. Numbers denote the fractions collected from top to bottom.
3.2.2 Mutations that generate leaky vesicles

Another class of mutations, e.g. E171A and D147A on S3 (Fig. 3.6C); and R205A, R208A and R211A on S4 (Fig. 3.6D), showed an initial decrease in fluorescence that was followed by a slow recovery. This may be explained by the mutations causing a membrane leak that allowed the passage of Na\(^+\) or Cl\(^-\), both of which are present in high concentrations in these experiments. After addition of valinomycin, the membrane potential was clamped at the equilibrium potential for K\(^+\) (approximately -60 mV, negative inside relative to outside, calculated with equation 1.1). This negative potential creates a driving force for the entrance of H\(^+\) into the vesicles. However, the negative electrical potential also creates a driving force for Cl\(^-\) to exit the vesicles (the concentrations of Cl\(^-\) is nearly symmetric across the vesicular membrane) and an even stronger driving force for Na\(^+\) entry (there is a large Na\(^+\) gradient across the membrane: 150 mM Na\(^+\) outside vs. ~0 mM Na\(^+\) inside). Therefore, if the mutation resulted in the generation of a leak for either of these ions, they would cross the membrane and dissipate the driving force for H\(^+\) uptake into the vesicles.

Depending on the rate of the leak, it might still be possible to see the fast influx of H\(^+\) followed by a slower dissipation of the H\(^+\) driving force caused by the leak, after valinomycin addition. This would result in the slow efflux of H\(^+\) from the vesicles and a slow recovery of the fluorescence signal (see below for a comparison between experiment and theory for leaky vesicles). To varying degrees, this is exactly what was observed for these mutant H\(_V\) channels (e.g. R205A in Fig. 3.5D), indicating that the mutation resulted in a Cl\(^-\) or Na\(^+\) leak through the VSD. If the leaky VSD were permeable to K\(^+\) in addition to Na\(^+\) or Cl\(^-\), a collapse of the K\(^+\) gradient would begin prior to addition.
of valinomycin, resulting in a lower overall fluorescence signal upon addition of valinomycin. This is what was observed for the R208A and R211A mutants (Fig. 3.6D). In fact, more recent electrophysiological experiments on R211 in hH₁ indicate that mutation to Ser or Cys results in the leak of the organic guanidinium cation through the channel (Berger & Isacoff 2011).

Mutations of VSDs that result in a leak current through the VSD itself (the so-called “omega current”) are relatively common and have been characterized in both Kᵥ and Naᵥ channels (Tombola et al. 2006; Sokolov et al. 2005). Given the delicate structural balance that the VSD must fulfill—compensating the charges of the gating residues within the low electrical permittivity of the membrane—it is not surprising that mutation of charged residues within the transmembrane domain leads to disruption of the structure, producing a leak pathway for the conduction of ions. It is interesting to note that all of the mutations that caused significant leak resulted from the removal of a charged amino acid side chain (Fig. 3.5C and D).

3.2.3 D112 an interesting position

A single mutation significantly slowed the H⁺ flux into the vesicles: D112L on S1 (Fig. 3.5A). Interestingly, an aspartate at this position on S1 is highly conserved in Hᵥ channels, but the equivalent position in the VSDs of other voltage-gated cation channels is a serine (Fig. 3.5; Musset et al. 2011). If this mutation only affected the rate of H⁺ conduction, we would expect that, given enough time to equilibrate, the same Hᵥ dependent fluorescence quenching (Fᵥ) would be observed when compared to wild type. However, even if the experiment is run over long time courses, the fluorescence
quenching seen in the D112L mutation never reached the same levels as wild type (Fig. 3.6B). Since the D112L reconstitution occurs efficiently (Fig. 3.6C), this indicates either that there are a larger proportion of non-functional channels in the vesicles, or that there is a leak current that results in partial dissipation of the electrical driving force, resulting in a stable steady state level of H⁺ uptake and fluorescence-quenching (see below for a theoretical analysis incorporating Cl⁻ leak).

To further investigate the potential role of D112 in the mechanism of H⁺ conduction, two additional mutations were made: D112S and D112A, both of which also displayed a significant decrease in H⁺ flux (Fig 3.6B). More recent electrophysiological experiments have demonstrated that mutation of D112 can make the channel leak Cl⁻ ions (Musset et al. 2011). In that study, both the D112S and D112A mutations were shown to have a strong Cl⁻-dependent shift in the reversal potential of the measured current, indicating Cl⁻ conduction through the Hᵥ channels (Musset et al. 2011). The D112L mutation was not investigated in that study; however, Musset et al. did generate D112N and D112F, both of which also showed Cl⁻ conduction (Musset et al. 2011). Therefore, it is likely that the D112L mutation also results in Cl⁻ leak, which would account for our observations in the flux assay that this mutant doesn’t show the same steady state level of Hᵥ dependent fluorescence quenching (Fₐᵥ) as wild type channels (Fig. 3.5A and 3.6B).

Because the D112L mutation alters H⁺ conduction through the channel in the flux assay (Fig. 3.5A and 3.6B) and the D112V mutation, examined by Musset et al., completely abolished conduction though the channel (Musset et al. 2011), it has been postulated that D112 is critical to both the conduction and selectivity of Hᵥ channels. However, whether the aspartate is sufficient remained unknown. To determine if an
aspartate at the equivalent position as D112 in S1 is sufficient to induce H⁺ flux through VSDs, we generated the S38D mutation in the isolated-VSD of KvAP (Fig. 3.7). As shown in Fig. 3.3, removal of the KvAP VSD from the pore domain revealed a possible intrinsic potential for H⁺ conduction: a slow H⁺ conduction was observed in the reconstituted isolated VSD. However, when we made the mutation S38D, we saw a significant increase in the rate of H⁺ conduction through the isolated VSD (Fig. 3.7). This indicates that an aspartate at this position on S1 is sufficient to promote H⁺ conduction through the VSD.
Fig. 3.7 D112 in S1 is sufficient for H\textsuperscript{+} conduction

Fluorescence-based H\textsuperscript{+} flux assay for vesicles containing the wild type (blue) and S38D mutant (brown) Kv\textsubscript{AP} isolated VSD compared to vesicles containing full-length Kv\textsubscript{AP} (green) channels, hH\textsubscript{V1} channels (cyan) and empty vesicles (orange). Each curve corresponds to the average of 3-4 repetitions with the error bars indicating the standard error of the mean. Valinomycin and CCCP are added at the indicated time points. Kv\textsubscript{AP} channels, Kv\textsubscript{AP} VSD, and Kv\textsubscript{AP} VSD S38D were expressed and purified according to published procedures (Jiang et al. 2003a). Reconstitutions were carried out as described in materials and methods with the following protein to lipid ratios (wt:wt) 1:100 (Kv\textsubscript{AP}), 1:200 (Kv\textsubscript{AP} VSD), and 1:200 (Kv\textsubscript{AP} VSD S38D).
3.3 Modeling $H^+$ Conduction Through Reconstituted $H_V$ Channels

3.3.1 Understanding the time course of fluorescence decay

Although we are unable to determine the $H^+$ conduction rate we can ask to what degree the time course of fluorescence decay is at least qualitatively consistent with our expectation based on theory. We simulated $H^+$ flux into a population of vesicles with a mean number of $\mu$ channels per vesicle assuming that a vesicle will have $n$ channels with subset $m$ facing outside-in with a frequency:

$$f(n,m) = \frac{n!}{m!(n-m)!} \left( \frac{1}{2} \right)^{\mu} \frac{\mu^n}{n!} e^{-\mu}$$  \hspace{1cm} (3.3)

Upon addition of valinomycin, the membrane potential is driven to very near the Nernst equilibrium potential for $K^+$ (about -60 mV inside calculated from equation 1.1) because the $K^+$ permeability exceeds $H^+$ conductance under all conditions in the simulation (see equation 1.2). We modeled $H_V$ voltage-dependent gating with a two-state Boltzmann function with midpoint activation voltage $V_{mid} = 40$ mV and valence 3 (Ramsey et al. 2006; DeCoursey 2008).

The change in internal $H^+$ concentration was calculated using the algorithm described in Moffat et al. with slight modifications (Moffat et al. 2008). To account for the voltage-dependent gating property of the $H_V$ channels, the proton flux ($J_{H}$) was calculated by equation 3.4, where $G_{H}$ is proton conductance, $V$ is the membrane potential, $E_{H}$ is the equilibrium potential for $H^+$, $F$ is Faraday’s constant, $z$ is the effective gating charge (a value of 3.0 $e_0$ was used; (DeCoursey 2008; Qiu et al. 2013; González et al. 2013).
2013), \( V_{\text{mid}} \) is the midpoint voltage of activation for \( H_v \) (a value of 40 mV was used; Ramsey et al. 2006), \( R \) is the ideal gas constant and \( T \) is the absolute temperature in Kelvin (298 K).

\[
J_H = \frac{G_H (V - E_H)}{F \left( 1 + \exp \left( \frac{-zF(V - V_{\text{mid}})}{RT} \right) \right)} \tag{3.4}
\]

The algorithm was run successively for a unit volume of one vesicle of radius 100 nm with \( n \) channels (where \( n = 1, 2, 3, \ldots, 30 \)), either facing outside-in or outside-out (expressed as a multiplier of either 1 or -1 on the \( V \) in the two-state Boltzmann). This basic set of 60 time courses representing the internal pH change of the vesicle was combined to generate the expected flux of a population of vesicles each containing \( n \) channels according to equation 3.5, where \( n \) signifies the total number of channels and \( m \) the number of channels facing outside-in.

\[
f(n,m) = \frac{n!}{m!(n-m)!} \left( \frac{1}{2} \right)^n \tag{3.5}
\]

Since the flux from channels facing outside-in is much greater than the flux due to channels facing outside-out (by more than 3 orders of magnitude), we applied the simplifying assumption that flux into any vesicle containing channels in both orientations was equal to the flux generated by only the channels facing outside-in. This operation results in a new basis set of 30 time courses that correspond to the \( H^+ \) flux into
populations of vesicles with total of n channels in either orientation (where n = 1, 2, 3, 30). This new basis set was then applied to the distribution of vesicles with n channels at the various protein-to-lipid ratios used according to equation 3.6, where \( f(n) \) is the fraction of vesicles with \( n \) channels, \( \phi \) is the fraction of functional H\(_V\) channels (a value of 1.0 was used), \( \theta \) is the fraction of reconstitution deficient vesicles (a value of 0.15 was used) and \( \mu \) is the ratio of number of channels to number of vesicles (see equation 3.1).

\[
f(n) = \left( \frac{\phi}{(1-\theta)} \right)^n \frac{\mu^n}{n!} \exp\left( -\phi \mu \right)
\]

All simulations were performed using MATLAB and the code can be found in Appendix I.

3.3.2 Comparing Experimental Results and Simulations

Fig. 3.8A graphs the simulation results for populations of vesicles with channels distributed according to equation 3.3. If one focuses on a single vesicle, a value 1.0 on the y-axis corresponds to a free (unbound) internal H\(^+\) concentration of 10\(^{-7}\) M and a value 0 corresponds to a free internal H\(^+\) concentration of 10\(^{-6}\) M. During the simulation, the free internal H\(^+\) concentration in the given vesicle changes from 10\(^{-7}\) to 10\(^{-6}\) (approximately), following a time course that depends on the number of channels and their orientation in the vesicle. The graph shows the weighted sum of time courses for all vesicles (including empty) in the population for a 200 s interval. The curves show a fast decay followed by a
slower one at smaller values of $\mu$: the slower component is attributable mainly to a fraction of vesicles with only outside-out channels, which have a very low open probability. The curves also show a negative second derivative (curvature) at early time points, due to $\text{H}^+$ buffering inside the vesicles. These same qualitative features are observed in fluorescence decay data (Fig. 3.8B).

![Graphs showing comparison of dilution series data with theory](image)

**Fig. 3.8** Comparison of dilution series data with theory

(A) Theoretical curves for the decrease of internal pH over time at the equivalent protein to lipid ratios as in Fig 3.2A, scaled with the theoretical fraction of empty vesicles. Curves are colored to match the equivalent experimental traces in Fig 3.8B; a theoretical curve corresponding to empty vesicles is not shown. Simulations were all performed using MATLAB. (B) Experimental fluorescence traces from Fig. 3.2A highlighting the first 200 seconds after the addition of valinomycin.
The theoretical and experimental curves are different in two obvious respects. As a function of $\mu$, the curves do not exhibit the same spacing between them. We think this most likely reflects the nonlinear (and unknown) relationship between free internal H$^+$ concentration and fluorescence (Fiolet et al. 1974). We emphasize that this unknown relationship prevents us from determining H$^+$ flux rates, but it does not prevent us from determining the fraction of vesicles with no channels versus vesicles with at least one channel (Fig. 3.2A and equation 3.2). The second difference between theory and experiment is a more prominent slow component of fluorescence change in the data, which is consistent with channel-independent H$^+$ leak in the vesicles (which we have not included in the model).

In the simulation, the curves correspond to an open channel conductance of 0.1 fS. This value should not be taken as an accurate determination of H$_V$ channel conductance for the reasons discussed above. However, this value is smaller than the reported conductance measured electrophysiologically – 10-100 fS (Cherny et al. 2003; DeCoursey 2008) – by a factor too large to be accounted for by the unknown relationship between H$^+$ concentration and fluorescence. One possibility is that the values used for $V_{\text{mid}}$ in our two-state Boltzmann distribution (equation 3.4) result in an overestimation of the open probability of the channel. Although these values were taken from the literature (Ramsey et al. 2006; DeCoursey 2008), it is well established that, due to H$^+$ depletion effects and the strong dependence of H$_V$ channel gating on the transmembrane pH gradient, it is difficult to get an accurate measure of $V_{\text{mid}}$ (DeCoursey 2008). Also, there is debate as to what the exact value of the valence of H$_V$ channels gating ($z$) is, with experimental values ranging 2-3 (Fujiwara et al. 2012; González et al. 2013). The
ambiguity in these values would only have a minor effect on the results from our model; however, they are sources of uncertainty that could contribute to underestimation of the unitary conductance. Additionally, our model treats each subunit of the dimeric H$_V$ channels as independently gating, whereas more recent data in the literature has established that the subunits behave cooperatively (González et al. 2010; Tombola et al. 2010; Musset et al. 2010b). Cooperativity would also affect the open probability of the channels in our simulation.

3.3.3 Simulating the effect of Na$^+$ and Cl$^-$ leak on H$^+$ uptake

Because some of the mutations we have investigated with the flux assay have been shown to cause leak through the hH$_V$1 channel (Berger & Isacoff 2011; Musset et al. 2011), we can ask how much does the time course of fluorescence signal observed for these mutants agree with theory incorporating a Na$^+$ or Cl$^-$ leak in the membrane? Na$^+$ and Cl$^-$ leak can be easily incorporated into equivalent circuit flux algorithm described in section 3.3.1 (and by (Moffat et al. 2008) by the addition of flux terms for the permeant ions. These flux terms are defined in equation 3.7 for Na$^+$ flux ($J_{Na}$) and equation 3.8 for Cl$^-$ flux ($J_{Cl}$), where $G_{Na}$ is the conductance of Na$^+$, $E_{Na}$ is the Nernst equilibrium potential for Na$^+$, $G_{Cl}$ is the conductance of Cl$^-$, $E_{Cl}$ is the Nernst equilibrium potential for Cl$^-$ and all other terms are defined as in equation 3.4.

$$J_{Na} = \frac{G_{Na}}{F} \left( V - E_{Na} \right)$$  \hspace{1cm} \{3.7\}
\[ J_{\text{Cl}} = -\frac{G_{\text{Cl}}}{F} (V - E_{\text{Cl}}) \]  \hspace{1cm} \{3.8\}

For simplicity the added Na\(^+\) and Cl\(^-\) leak conductances were modeled as independent of H\(_V\) channel gating. Versions of the algorithm that incorporated H\(_V\) channel state dependence into the leak currents did not alter the shapes of the curves calculated but only slightly altered the kinetics and final steady state positions for the theoretical pH\(_i\). This modified algorithm can be found in Appendix I.

Fig. 3.9A shows theoretical curves calculated from the algorithm used for simulation of the H\(^+\) flux into the vesicles with the addition of Na\(^+\) leak. These curves recapitulate the main features of the data for the leaky mutants E171A, D147A, R205A and R208A (compare Fig. 3.5C and D to Fig. 3.9A). Additionally, by increasing \(g_{\text{Na}}\) in the simulation, theoretical curves similar to the data for R211A can also be produced. Fig 3.9B shows the theoretical curve for the simulation of H\(^+\) flux into the vesicles with the addition of Cl\(^-\) leak. The theoretical curve in Fig. 3.9B (bottom) differs from the data in that the H\(^+\) flux reaches a steady state more quickly than what is seen in the D112L mutant fluorescence data. This discrepancy can be understood if the D112L mutation also decreased the H\(^+\) conductance. In order to reduce bias, the H\(^+\) conductance was kept the same as for the simulations in Fig 3.8 during the leak simulations (Fig. 3.8).
Fig. 3.9 Comparison of mutant data to theory incorporating leak conductances

(A) Comparison of R205A mutant channel flux data (top) to theoretical curves for the decrease followed by slow recovery of internal pH over time incorporating a Na$^+$ conductance ($G_{Na}$) in the membrane of 0.05 fS scaled with the theoretical fraction of empty vesicles (0.15, bottom). Simulations were all performed using MATLAB.

(B) Comparison of D112L mutant channel flux data (top) to theoretical curves for the decrease of internal pH over time incorporating a Cl$^-$ conductance ($G_{Cl}$) in the membrane of 10 fS scaled with the theoretical fraction of empty vesicles (0.15, bottom). Simulations were all performed using MATLAB according to the protocol described in section 3.2 with protein-to-lipid ratios of 1:100 (wt:wt).
3.4 DISCUSSION AND FUTURE DIRECTIONS

The data presented in this chapter clearly establish the ability of the putative hHv1 channel to conduct H⁺ (Lee et al. 2009b). Furthermore, through mutagenesis studies on the transmembrane domain, we identified an interesting amino acid residue position—D112 on S1— which, when mutated, impairs the channel’s ability to conduct H⁺. This finding has since been confirmed electrophysiologically by Musset et al., who demonstrated that mutation of this position can lead to Cl⁻ leak through hHv1 and that the D112V mutant is incapable of conducting ions (Musset et al. 2011). By mutating the equivalent position on S1 of the KvAP isolated VSD to aspartate (S38D), we demonstrated that an aspartate at this position is sufficient to promote H⁺ conduction through VSDs.

Going forward, it would be interesting to generate, purify and reconstitute the D112V mutant and to test it for H⁺ flux in our assay. Given that Musset et al. failed to see any conduction through the D112V mutant channel even though it was efficiently expressed to the cell membrane (Musset et al. 2011), we would expect that this mutant would completely abolish H⁺ flux in our assay. In order to fully understand the role of D112 in conduction, it would be ideal to solve the structure of the channel in both the conductive and non-conductive states. Because of the delicate charge balance that is found in VSDs, one may expect that mutations of D112 to an uncharged residue may disrupt the protein’s stability. However, given that the D112L mutant channel displayed greater stability in detergent than wild type hHv1 in our expression and purification experiments, we believe that the D112L mutant may constitute a good structural target.
Many of the mutants we characterized in our flux assay show behavior consistent with the generation of a leak conductance through the membranes (Fig. 3.9). However, in order to confirm whether these mutations cause the production of leaky VSDs these mutants will have to be expressed in cell membranes and characterized electrophysiologically. The cases in which the indicated mutants or similar mutants have been characterized electrophysiologically have demonstrated that leak currents exist through the channel (Berger & Isacoff 2011; Musset et al. 2011).
CHAPTER 4: CRYSTALLOGRAPHY

One major goal of my doctoral work was to obtain the structure of the human voltage-gated H⁺ channel (hHᵥ1), in order to shed light on the unique properties of Hᵥ channels. Although structures may not provide direct mechanistic insight, they do provide a model for hypothesis generation, which can then be followed up by functional studies. Before I joined the lab, Dr Seok-Yong Lee had done initial construct design and crystallization screening on hHᵥ1. My work described here follows from Dr Lee’s initial work, and we worked as a team for the first three years.

In our efforts to crystallize the hHᵥ1 channel, Dr Lee and I generated many different constructs (discussed below), which were tested for expression and biochemical stability. Biochemical stability here is defined as monodispersity on a size exclusion chromatography (SEC) column after remaining in solution undisturbed at high concentrations for 5-7 days, as monodispersity on SEC has been shown to correlate well with crystallizability (Kawate & Gouaux 2006). Only biochemically stable constructs were pursued in crystallization trials.

In addition to hHᵥ1, 21 other putative Hᵥ channel genes were synthesized and screened for biochemical stability. Appendix II contains the sequences of all genes synthesized and shows a multiple sequence alignment between all of the putative Hᵥ channel genes as well as the voltage sensor domain of rat Kᵥ1.2. Dr Scott Hansen a
postdoctoral fellow in the lab joined Dr Lee and I in this effort, which unfortunately failed to identify any other promising functional targets. The putative H_v channel from the plant genus *medicago* was biochemically well behaved but when reconstituted into lipid vesicles did not result in any H^+ conduction and was therefore set aside.

### 4.1 Native and Fusion Protein Crystallographic Attempts

#### 4.1.1 Crystallography of wild type truncated channels

Despite many attempts, crystals of the full-length hH_v1 never grew. This is not surprising, given that the acid-and-proline-rich N-terminus of hH_v1 is predicted to be disordered. To increase crystallizability by removal of the unstructured regions, Dr Lee worked with Dr Qingjun Wang (Laboratory of Mass Spectrometry and Gaseous Ion Chemistry at the Rockefeller University) to define the structural core of the hH_v1 channel by limited proteolysis and by mass spectroscopy. Upon the identification of two trypsin-sensitive sites (at amino acid R83 in the N-terminus, preceding the transmembrane domain, and at K221, between the transmembrane domain and the coiled coil), Dr Lee designed two truncated constructs: one with the C-terminal coiled coil removed (hH_vΔC) and one with both N- and C-termini removed (hH_vΔNΔC).

Expressing these constructs in *Pichia pastoris* and using the high affinity 1D4-tag for purification, Dr Lee was able to purify both constructs and grow small crystals of the hH_vΔNΔC construct (Fig. 4.1A). These truncated channels crystallized under many conditions; however, none diffracted better than ~10 Å (Fig. 4.1B). Nevertheless, this was encouraging, since similar diffraction had been previously observed for the isolated
voltage-sensor domain (VSD) of the voltage-gated K⁺ channel KᵥAP. When an antigen-binding fragment (Fab) of an antibody raised against the isolated VSD of KᵥAP was used as a crystallization chaperone, Jiang et al. were able to solve the structure of isolated VSD to 1.9 Å resolution (Jiang et al. 2003a). Therefore, by analogy, we reasoned that, if we could obtain a Fab against the hHᵥ1 channel and use it in crystallization, we should be able to obtain crystals with improved diffraction.

Fig. 4.1 Best diffracting crystals of hHᵥΔNΔC

(A) Representative crystals of hHᵥΔNΔC grown in DM/HEGA9 detergent at 30% PEG400, 50 mM NaAcetate pH 4.5. (B) Best diffraction from crystals shown in (A), resolution extends to 9.6 Å, apparent space group is F23 a=b=c=169.7 Å. Weak diffraction is also seen for a unit cell with an alternate orientation indicating possible twinning. These crystals were grown and their diffraction tested by Dr Seok-Yong Lee.
4.1.2 Raising antibodies against hHv1

We attempted raising antibodies against both the wild type and truncated (hHvΔNΔC) hHv1 channels by mouse injection. The only anti-hHv1 antibodies that were isolated were against an intracellular C-terminal coiled-coil epitope, which, according to the limited proteolysis studies, is connected to the transmembrane domain by a flexible linker. Although these antibodies worked well in Western blots and were used for the stoichiometry studies discussed in Chapter 2, no crystals of hHv1-Fab complex grew in our crystallization trials. Given that Fabs that were successful for the crystallization of the KvAP isolated VSD were targeted towards an extracellular loop of the transmembrane domain (the S3-S4 helix-turn-helix motif known as the voltage-sensor paddle), an anti-hHv1 antibody that targeted the voltage-sensor paddle of hHv1 was desirable. An additional round of mouse injections was performed with the hHvΔNΔC doubly truncated construct, with injections of the channel reconstituted in lipid vesicles, followed by boosters of detergent-solubilized channels. As in the wild type case, we were unable to isolate any anti-hHv1 antibodies.

Because mouse injection has been very successfully applied in the MacKinnon laboratory for raising antibodies against membrane proteins (Zhou et al. 2001; Dutzler et al. 2003; Jiang et al. 2003a; Brohawn et al. 2013), a purely technical reason for our failure was unlikely. A possible explanation is that, given the human and mouse Hv channel sequence-similarity (78% identical over the entire sequence and 84% identical within the transmembrane domain alone), the hHv1 channel may not be sufficiently immunogenic in mice to produce a strong antibody response.
In order to address this possible issue, we attempted another round of mouse injections using chimeric channels that contained the voltage-sensor paddle of \( hH_v 1 \) spliced into the \( K_v \text{AP} \) isolated VSD, reasoning that the \( H_v \) sequence presented in this context should increase the immunogenicity. We built and characterized six chimeras based on three possible \( K_v \text{AP}/hH_v 1 \) alignments (Fig. 4.2A and B). Although the chimeric channels expressed well and were biochemically well behaved (Fig. 4.2C), they were not sufficiently immunogenic for us to be able to isolate any anti-\( hH_v 1 \) Fabs.

More recent work in the literature has demonstrated that \( hH_v 1 \) channels play a physiological role in B-cell receptor signaling: \( hH_v 1 \) knockdown in B-cells results in impaired antibody production (Capasso et al. 2010). Thus, it is likely that there is a negative selection against B-cells that produce high-affinity anti-\( H_v 1 \) antibodies that are able to bind to extracellular epitopes. Such negative selection is a possible explanation as to why we were unable to raise any antibodies by mouse injection.

Despite these setbacks, we still reasoned that it should still be possible to improve the diffraction of \( hH_v 1 \) crystals with a crystallization chaperone. Two additional strategies were attempted to this end: fusion proteins and epitope-swapped chimera. The fusion protein strategy was ineffective and I will only discuss it briefly below. The epitope-swapped chimera will be more fully discussed in the following section.
Fig. 4.2 Sequences and biochemical stability of $K_v$AP VSD-hH$_v$1 paddle chimeras

(A) Three possible registers for sequence alignments between the S3-S4 paddle motif of $K_v$AP (in black) and hH$_v$1 (in blue). The numbers above the $K_v$AP sequence denote the positions of the gating-charge arginines (highlighted red in all sequences) for the first alignment. The alignments are named based on the relative position of the first hH$_v$1 gating-charge arginine with respect to the $K_v$AP gating-charge arginines. For example, alignment 1-1 aligns the first arginine of hH$_v$1 with the first arginine of $K_v$AP, alignment 1-2 aligns the first arginine of hH$_v$1 with the second arginine of $K_v$AP etc. (B) Paddle sequences of the six chimeric (APH$_v$) constructs built, organized by the alignment that was used for their construction. (C) SEC profiles of each chimeric construct organized by alignment. Each chromatogram contains the $K_v$AP VSD curve (blue) to allow for comparison of expression levels. From these data it is clear that, although all constructs are monodisperse, we are able to purify larger quantities of APH$_v$3 and APH$_v$4 from the 1-2 alignment.
4.1.3 Lysozyme Fusion Proteins

The fusion of the highly crystallizable enzyme lysozyme into the transmembrane domain loops of membrane proteins has been used as a chaperone strategy to crystallize difficult membrane protein targets. For instance, this strategy was used to determine the structure of the first G-protein coupled receptor (GPCR; Rosenbaum et al. 2007). We attempted this strategy by splicing lysozyme into both extracellular loops of the hHvΔNΔC construct (the S1-S2 and S3-S4 loops). At low concentrations, the lysozyme fusions looked promising; however, they readily aggregated at high concentration. Optimization of linker length between the hHv1 and lysozyme sequences was carried out, but no construct was sufficiently well behaved for large-scale crystallization attempts. Because of this tendency for aggregation, the lysozyme fusion strategy was abandoned.

4.1.4 Dimerization Fusion Proteins

Since we knew from our cross-linking studies that hHv1 is a dimer in the membrane, we reasoned that by fusing readily crystallizable dimeric protein onto hHv1 we might be able to promote crystallization. A major criterion for the fusion partner would be for it to have its N- or C-terminus arranged in the dimer such that it would be positioned to align with our experimental model of the hHv1 dimer (see Fig. 2.5 on page 48). Four top candidates were identified from the protein data bank: secretion chaperone CsaA (accession code 2NZH; Shapova & Paetzel 2007), F420H2:NADP+ oxidoreductase (FnO; accession code 1JAX; Warkentin et al. 2001), the bleomycin resistance determinant (Bleo; accession code 1ECS; Maruyama 2000) and 4-diphosphocytidyl-2-C-methylerythritol synthetase (CDP; accession code 1INJ; Richard et al. 2001). Two of these proteins were selected for
N-terminal fusion (FnO and CDP) and two for C-terminal fusion (Bleo and CsaA) to the hHvΔNΔC construct. For each fusion, four or five different constructs were built to optimize linker length. After much effort, however, and although some of the constructs generated well-behaved dimeric chimera, no fusions gave crystals. Therefore, this approach was also abandoned.

4.2 Hv1-KvAP Epitope-Swapped Chimera

4.2.1 Design and selection of Hv1-KvAP paddle chimera

As discussed in the previous section, by analogy to the KvAP isolated VSD, we reasoned that Fab-mediated crystallization would facilitate structure determination of hHvΔNΔC. Since we were unable to raise antibodies against wild type hHv1 channels by mouse injection or to obtain crystals by the fusion protein strategy, we needed to innovate. It occurred to us that it should be possible to make a hHv1-KvAP (HAP) chimera in which the paddle epitope that is responsible for binding anti-KvAP Fabs is spliced into the hHv1 sequence. It has been previously demonstrated that the paddle motif can be swapped between different VSD of voltage-gated cation channels and still produce functional voltage-gated cation channels (Alabi et al. 2007). In this way, we could generate functional chimeric Hv channels that would bind to the anti-KvAP Fabs, allowing us to use the anti-KvAP Fabs as crystallization chaperones.

Due to the ambiguity in the sequence alignment between hHv1 and KvAP along the S4 helix, many different constructs needed to be built and characterized before crystallization trials could proceed. Based on three different possible hHv1 and KvAP S4
alignments, 17 chimeras were constructed (Fig. 4.3) and each was expressed in both full length and C-terminally truncated forms (ΔC). Small-scale purification and biochemical characterization using size exclusion chromatography (SEC) was carried out to gauge the stability of each chimeric channel. Two chimeras each with different alignment—HAP5ΔC and nHAP3ΔC—were chosen as the best candidates for structure determination, based on their biochemical stability and Fab-binding. When probed for binding with the anti-KvAP antibodies 33H1 and 6E9, both chimeras were ELISA- and Western-blot-positive, indicating that the antibodies were able to recognize the paddle epitope of the chimeric constructs (Fig. 4.4).

The HAP5ΔC chimera was highly stable, giving a stable monodisperse peak on the SEC column after several days at high concentrations at room temperature in DM (Fig. 4.5A-C). However, when mixed with anti-KvAP Fabs and run over the SEC column, only a small complex peak was formed, with most of the protein eluting at positions characteristic of the channel and Fab alone (Fig 4.5D and E). This is in contrast to what is observed with wild type KvAP channel or isolated VSD, which elute completely as VSD-Fab complex. This indicated that, although HAP5 is ELISA-positive for binding to 33H1 and 6E9 Fabs (Fig. 4.4), the interaction is much weaker than the binding of the Fabs to the wild type KvAP VSD.
Fig. 4.3 HAP chimera construct sequences

(Top) Three possible registers for sequence alignments between the S3-S4 paddle motif of \( \text{K}_\text{V} \text{AP} \) (in black) and \( \text{hH}_\text{V} \text{1} \) (in blue). The numbers above the \( \text{K}_\text{V} \text{AP} \) sequence denote the positions of the gating-charge arginines (highlighted red in all sequences) for the first alignment. The alignments are named as in Fig. 4.2. The first 16 HAP chimeras were generated and characterized by Dr. Seok-Yong Lee and were all based on the 1-3 alignment. The “New Chimera” designated with an “n” were constructed based on the 1-2 and 1-1 alignments.
Fig. 4.4 ELISAs and western blots of chimeras using αKVAP paddle antibodies

(A) Bar graphs of ELISA signal intensity as a function of Ab dilution (indicated on right) for KVAP VSD (cyan), nHAP2ΔC (red), nHAP3ΔC (yellow), HAP5ΔC (green) and hHv1 (purple) using the αKVAP monoclonal antibodies 6E1 (left) and 33H1 (right). The low signal seen for the KVAP VSD is due to poor binding of this protein to the plates. (B) SDS-PAGE gels and corresponding western blots against the indicated chimera using the αKVAP monoclonal antibodies 6E1 (left and center) and 33H1 (right).
Fig. 4.5 Biochemical stability of HAP5ΔC and binding of αKvAP paddle antibodies
(A) SEC chromatogram of rerun single concentrated fraction after initial SEC purification. (B) SEC chromatogram of pooled fractions from initial SEC run concentrated and left overnight (O/N) at room temperature (RT). (C) SEC chromatogram of pooled fractions from initial SEC run concentrated and left for 4 days at RT. (D) SEC chromatogram of HAP5ΔC with 6E1 Fab indicating some weak complex formation in DM. (E) SEC chromatogram of HAP5ΔC with 33H1 Fab indicating no significant complex formation. All SEC runs were done in DM.
The nHAP3ΔC chimera was less stable than the HAP5ΔC construct; nHAP3ΔC readily aggregated at high concentrations in DM (Fig 4.6A and B). However, in the more dispersive detergent LDAO nHAP3ΔC was more stable (Fig4.6C-E) and it eluted completely as complex with the anti-KvAP 6E1 Fab on the SEC column (Fig. 4.6F), similar to the wild type KvAP VSD. Because of the differences in the biochemical behavior of these two chimeras, it was decided that both would be pursued for structure determination.

In order to explore possible crystallization conditions, the detergent-stability profiles of the two chimeras was examined. After purification of the constructs in decylmaltoside (DM), small volumes of the protein were injected onto a SEC column equilibrated in the detergent of interest. Stability in the new detergent was judged based on the monodispersity of the protein peak eluting from the SEC column. In this way, 19 detergents were examined (Table 4.1). Consistent with the differential stability of the chimeras in DM (see Fig. 4.5A-C vs. 4.6A and B), the HAP5ΔC chimera was stable in a diverse array of detergents, whereas the nHAP3ΔC chimera was only highly stable in dispersive and lipid-like detergents (Table 4.1).
Fig. 4.6 Biochemical stability of nHAP3ΔC and binding of αKvAP paddle antibodies
(A) SEC chromatogram of rerun single concentrated fraction after initial SEC purification in DM. (B) SEC chromatogram of pooled fractions from initial SEC run concentrated and left O/N at RT in DM. (C) SEC chromatogram of detergent exchange from DM to LDAO. (D) SEC chromatogram of pooled fractions from (C) concentrated and left O/N at RT in LDAO. (E) SEC chromatogram of pooled fractions from (C) concentrated and left for 5 days at RT in LDAO. (F) Reducing SDS-PAGE gel (left) of SEC chromatogram (right) fractions for nHAP3ΔC with 6E1 Fab indicating complex formation in LDAO (right)
Table 4.1 Detergent stability profiles of HAP5ΔC and nHAP3ΔC chimeras

<table>
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<th>Detergent</th>
<th>HAP5DC</th>
<th>nHAP3DC</th>
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<td>Glucosides</td>
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<th>Detergent</th>
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<td>FoS-12</td>
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<td>Zwittergent 3-12</td>
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<tr>
<td>HEGA-9</td>
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<td>LPPG</td>
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Detergent stability is graded qualitatively from A to F based on peak monodispersity during detergent exchange experiments on SEC. A dash (-) indicates that the experiment was not done. Inset shows an example A grade (left) vs. an example F grade (right).

Given the diversity of detergent conditions in which the chimeras were stable, many conditions needed to be explored for crystallization. Initial crystallization trials of HAP5ΔC and nHAP3ΔC with the 6E1 anti-K\textsubscript{v,AP} Fab gave small crystals in the case of the HAP5ΔC construct in DM (Fig. 4.7), but did not give any crystals in the case of the nHAP3ΔC in DM, LDAO or DHPC. However, crystalline precipitate was seen in many drops of the nHAP3ΔC/6E1 crystallization screens, indicating that it might be possible to further optimize the conditions to produce crystals. For HAP5ΔC, I was unable to
successfully refine the initial hits from 6E1 crystal screens, suggesting that alterations to the construct might be required to facilitate efficient crystallization.

Although the C-terminally truncated wild type hH\textsubscript{V1} (hH\textsubscript{V1}\textsubscript{ΔC}) construct gave some crystals, truncation of both the N- and C-termini improved crystallizability (Fig. 4.1). Therefore, we reasoned that removal of the N-terminus might also be required to improve crystal formation of the chimeric constructs, as described below.

Fig. 4.7 Initial crystal hits for HAP5\textsubscript{ΔC} in complex with 6E1 Fab
Bright field (left) and UV emission (right) images of small crystals that grew in drops containing HAP5\textsubscript{ΔC}/6E1 in DM with 10-30\% PEG400, 200 mM CaCl\textsubscript{2}, 50 mM NaAcetate, pH 4.5. The fluorescent signal seen in the UV emission image indicates that the crystals are proteaceous.
4.2.2 Construct optimization and improvement of the initial crystals

In order to optimize the chimeric constructs in an unbiased manner, we performed limited proteolysis on both HAP5ΔC and nHAP3ΔC. In this way, any differences in the protease-resistant regions of the constructs could be examined and compared to wild type hH\(_{\nu}\)1 channels. By defining the protease resistant core of the chimeras, we would be able to redesign the constructs to remove any flexible or unstructured regions that might be interfering with crystallization.

Limited proteolysis was carried out for each chimera using four proteases: trypsin, chymotrypsin, elastase and subtilisin (Fig. 4.8). For both chimera, one hour of trypsinization resulted in the smallest stable fragment. These fragments were then analyzed by both N-terminal sequencing and mass spectrometry, which revealed that the trypsin cleavage site was located between amino acids R83 and A84. This is the same position at which limited proteolysis removes the N-terminus of the wild type hH\(_{\nu}\)1 channel, indicating that the beginning of the structured transmembrane domain is unaltered in the chimeric constructs.
Fig. 4.8 Example limited proteolysis experiments for nHAP3ΔC in DM

(A) nHAP3ΔC was mixed with the indicated proteases at ratios ranging from 0.0001 mg protease/mg channel to 0.1 mg protease/mg channel for 1 hour at room temperature. Digestions were stopped by addition of 5 mM PMSF. (B) Western blot analysis of the proteolysis fragments generated at the highest protease concentrations in (A). SDS-PAGE gel (left) indicating the presence of each of the fragments as well as wild type hHv1 and KvAP VSD controls. Western blot (middle) using the αKvAP-paddle mAb 6E1 as primary Ab indicating that each proteolysis fragment contains the paddle epitope indicating that the transmembrane region is resistant to proteolysis. The contents of each well are indicated in the legend (right). Trypsin fragment was further characterized by N-terminal sequencing and mass spectrometry.
After initial proteolysis experiments, a trypsinization time-course was carried out to compare the relative stabilities of HAP5ΔC and nHAP3ΔC (Fig. 4.9). These experiments indicated that the trypsin-digested HAP5ΔC was highly stable: no further proteolysis was observed after the initial cleavage even at very high trypsin concentrations (Fig. 4.9B). In contrast, the nHAP3ΔC continued to degrade over time, indicating instability (Fig. 4.9A). However, by stopping the trypsin digestion of nHAP3ΔC at an early time point by addition of phenylmethanesulfonylfluoride (PMSF), it was possible to isolate the transmembrane fragment before it was further digested (Fig. 4.9A).

Since it was possible to isolate a stable transmembrane fragment of both chimeric constructs, preparative trypsinization was attempted as a possible method for protein production for crystallization. Using this methodology, the protein was digested by addition of trypsin during the purification, the digestion was stopped by addition of PMSF, the transmembrane fragment was isolated by SEC, mixed with the anti-KvAP Fabs (6E1 or 33H1) and then set up in crystallization screens. This process resulted in the growth of crystals of trypsinized-HAP5ΔC (tHAP5ΔC) in complex with 6E1. No crystals were observed for the nHAP3ΔC construct with either 6E1 or 33H1 Fabs.
Fig. 4.9 Trypsinization time-course to compare stability of chimeric constructs

(A) nHAP3ΔC in LDAO and (B) HAP5ΔC in DM were subjected to trypsinization at the indicated trypsin-to-channel ratios (bottom of gels). At the indicated time points an aliquot was taken and quenched with PMSF then run on the SDS-PAGE gel. LDAO was used for nHAP3ΔC since the channel is more stable in this detergent than in DM (see Table 4.1).
Refinement of the tHAP5ΔC/6E1 crystallization conditions resulted in the growth of large (~0.4 x 0.2 x 0.1 mm) crystals (Fig. 4.10A). In order to confirm that these crystals contained both channel and Fab, crystals were harvested, washed and run on an SDS-PAGE gel, which was developed using silver staining (Fig. 4.10B). This gel indicated that the crystals contained both Fab and tHAP5ΔC (Fig. 4.10B), demonstrating that, even though the interaction between this construct and the Fabs are too weak to purify complex on the SEC column, complex does form under crystallization conditions.

The X-ray diffraction properties of these tHAP5ΔC/6E1 crystals were examined at the synchrotron. The crystals diffracted anisotropically to ~3.5 Å in the good direction and ~9 Å in the other two directions (Fig. 4.10C). Although I was unable to index this data set, it was encouraging to see diffraction from these crystals. We decided it would be best to focus efforts on improving this crystal form and to forego working on the nHAP3ΔC chimera, which had yet to produce any crystals.
Fig. 4.10 Crystals of trypsinized HAP5ΔC in complex with 6E1

(A) Crystals of trypsinized-HAP5ΔC (tHAP5ΔC) in complex with 6E1 Fab grown in 30% PEG400, 50 mM potassium phosphate pH 7.0. (B) Silver stained SDS-PAGE gel of crystals from these conditions indicating that the crystals contain both 6E1 Fab and tHAP5ΔC. (C) Best diffraction pattern generated by crystals from these conditions.
To improve the diffraction, I needed to eliminate any source of heterogeneity originating from the protein preparation. To generate channel protein for these crystals, the construct was expressed as a C-terminal GFP-1D4 fusion, with the channel and GFP-1D4 tag separated by a PreScission protease cleavage site. The channel was purified by 1D4-affinity chromatography followed by PreScission protease digestion to remove the GFP-1D4 tag, followed by an additional round of 1D4-affinity chromatography to separate the cleaved GFP-1D4 from the channel, trypsinization to remove the N-terminus and then SEC to isolate the transmembrane fragment. PreScission protease is a highly specific protease isolated from human rhinovirus that cleaves the sequence LEVLFQ/GP where the “/” indicates the site of cleavage (Cordingley et al. 1990). Because of the eight amino acid residue long recognition site and an additional three amino acid residues introduced for cloning purposes, removal of the C-terminal GFP-1D4 tag by PreScission protease resulted in the presence of nine non-H\textsubscript{\textalpha} derived amino acid residues (SNSLEVLFQ) following the final native lysine residue (K221 in wild type channels) on the C-terminus of the HAP5ΔC construct. Mass spectrometry of the trypsinized channel indicated that not only did the trypsin cut at position R83 on the N-terminus, but it also cut much more slowly at the final native lysine residue, producing a mixture of C-termini (Fig 4.11). We reasoned that removal of this source of heterogeneity by redesign of the expression construct should improve crystal growth.
Fig. 4.11 Mass Spectrometry of HAP5ΔC trypsinization time course

(A) Mass spectrum of undigested HAP5ΔC generated using the ultrathin-layer method (Cadene & Chait 2000). The experimental mass agrees well with the theoretical mass for the full-length construct with the N-terminal methionine removed. The additional ~42 Da mass difference between the theoretical and experimental masses indicates that the protein contains a post-translational modification, most likely N-terminal acetylation. (B) Mass spectrum of HAP5ΔC after 3 hours of trypsin digestion indicating that the N-terminus has been completely removed by trypsin digestion at R83. Two distinct peaks corresponding to the transmembrane (TM) fragment are visible. The masses of the two fragments TM1 and TM2 correspond to within 1 Da of the theoretical masses of the HAP5ΔC TM fragment plus/minus the nine non-native amino acid residues left over from the PreScission protease site on the C-terminus. (C) Mass spectrum of HAP5ΔC after overnight trypsin digestion at 0.1 mg trypsin/mg channel indicating that given enough time the nine non-native amino acid residues are completely removed.
To bypass preparative trypsinization, the GFP tag was moved to the N-terminus, making the PreScission site N-terminal to the transmembrane domain. Since the N-terminal site of trypsinization was located between R83 and A84, through overlapping of the PreScission cleavage site with this trypsin site, it was possible to generate a final PreScission-digested N- and C-terminally truncated chimera that only differed from the wild type HvΔNΔC by having a glycine instead of an alanine at position 84 and by terminating at the lysine position 221 (and, of course, by having the KvAP paddle swap). However, because the 1D4 affinity tag is C-terminal, moving the tag to the N-terminus required using a different affinity purification protocol. At first, an N-terminal FLAG tag was used, which also allows antibody affinity for the purification of low-yield proteins. However, after initial expression and purification trials, it was found that moving the GFP tag to the N-terminus greatly improved the protein expression and more than doubled the yield. Therefore I was able to switch the affinity tag from a FLAG tag to a Deca-His tag and thus use immobilized metal-affinity chromatography (IMAC) in the purification.

By making the above modifications to the expression construct, I was able to simplify the purification protocol by removing the need for preparative trypsinization. With this construct, the purification protocol after protein extraction consisted of IMAC using Co^{2+} resin, PreScission digestion to cleave off the His-GFP tag, another round of IMAC to separate the cleaved tag and the HAP5ΔNΔC construct, followed by a final SEC purification. Mass spectrometry confirmed that this new construct and purification protocol resulted in the production of a homogenous protein sample without any additional proteolysis or post-translational modifications.
Setting up crystallization screens with this construct in the presence of the 33H1 Fab in DM resulted in two new crystal forms both of which contained Fab and HAP5ΔNΔC (Fig 4.12). After refinement of the crystal growth conditions and initial diffraction analysis, it was determined that the crystal form shown in Fig. 4.12A did not diffract better than ~9 Å resolution, whereas the other crystal form, shown in Fig. 4.12B, displayed greater variability with some crystals diffracting better than 4 Å. Screening of many different crystals from these conditions resulted in collection of a complete, though anisotropic, data set to 3.9 x 5.1 x 3.8 Å resolution (Fig. 4.13, Table 4.2).

Before discussing the structure of HAP5ΔNΔC that I obtained from this data set, I will use the following section to describe the functional work that was performed in conjunction with the biochemistry described in the previous sections. Throughout the design and modification of the chimeras, great care was taken to insure that each new construct was a functional voltage-gated proton channel. The functional data presented in the next section are essential for interpretation of the structural work.
Fig. 4.12 Crystals grown from HAP5ΔNΔC construct in complex with 33H1 Fab

(A) Crystals grown in 30% PEG400, 50 mM MES pH6.5, 100 mM NaLiSO₄. (B) Crystals grown in 30% PEG400. The silver stained gels of the crystals (right) indicate that the crystals contain both 33H1 Fab and HAP5ΔNΔC.
Fig. 4.13 Diffraction from crystals of HAP5ΔNΔC/33H1 complex crystals
Two diffraction images from the same crystal grown in 30% PEG400 taken at ~90° separation clearly showing anisotropy. The concentric circles indicate resolution shells, going from the center outwards 20 Å, 8 Å and 4 Å are shown.

Table 4.2 Summary of data collection for HAP5ΔNΔC/33H1 complex

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<th>Data Set</th>
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<tbody>
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<td>Source</td>
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<tr>
<td>Cell Dimensions: a, b, c (Å)</td>
<td>99.8, 136.1, 148.1</td>
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<td></td>
<td>α, β, γ (°)</td>
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<tr>
<td>Unique reflections</td>
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</tr>
<tr>
<td>I/σI</td>
<td>7.33 (2.47)</td>
</tr>
</tbody>
</table>

Ellipsoid truncation and anisotropic scaling was performed on the data set using the UCLA MBI diffraction anisotropy server (Strong et al. 2006).
4.2.3 The best candidates for structure determination are functional

By reconstituting the chimeric channels into lipid vesicles and using the fluorescence-based concentrative uptake flux assay described in Chapter 3, I determined that HAP5ΔC, nHAP3ΔC and the trypsinized HAP5ΔC were able to conduct H⁺ (Fig. 4.14A and B). In each case, the H⁺ conduction observed from these constructs was qualitatively similar to that of the reconstituted wild type hHv1 channel (Fig. 4.14A and B). The fact that the fluorescence quenching time courses went to completion without any slow recovery or intermediate level of quenching indicated that the chimeric constructs also maintained H⁺ selectivity (see Chapter 3 for a more in depth discussion on implications concerning selectivity from this assay). These functional data from the flux assay were sufficient for me to pursue these chimeric constructs as possible structural targets; however, once a complete data set was obtained for the HAP5ΔNΔC, a more rigorous electrophysiological characterization was desired.
Fig. 4.14 H$^+$ uptake into vesicles containing the chimeric constructs
(A) Fluorescence-based H$^+$ flux assay for vesicles containing HAP5ΔC (green), nHAP3ΔC (red) compared to empty vesicles (orange) and wild type hH$_V$1 containing vesicles (cyan). (B) Fluorescence-based H$^+$ flux assay for vesicles containing HAP5ΔC (green) and trypsinized-HAP5ΔC (tHAP5ΔC, brown). In each case valinomycin was added after 150s, CCCP was added at 450s and the fluorescence measurements were repeated 3-4 times (error bars represent the standard error of the mean). The significant fluorescence quenching seen for the empty vesicles in (B) indicate that this batch of vesicles was more leaky than usual.
To test the voltage-gating properties of the HAP5 chimera, the full-length sequence was cloned into a HEK-cell expression vector and the activity of the channel was compared to wild type hHv1 in HEK cells by the whole-cell patch clamp method. Although both channels generated robust voltage-gated currents, the gating properties of the HAP5 chimera are significantly different from that of hHv1 channels (Fig. 4.15A and B). Both the rate of opening and closing were significantly faster in the HAP chimeric channel (Fig. 4.15B). The rate of closing of the HAP5 chimera was so fast that, when using a similar voltage step protocol as for hHv1, tail current was not observable (Fig. 4.15B). Due to the speed of channel closing when the voltage was stepped to negative potentials, the reversal potential of the currents elicited by opening of the HAP5 channel could not be accurately measured. This precluded definitive determination of the ion selectivity for this channel; however, as mentioned above, the stability of fluorescence quenching seen in the flux assay suggests that the HAP5 channel maintained its H⁺ selectivity.

Further characterization of the chimeric channel under different external and internal pH and ionic conditions may have allowed for accurate measurement of reversal potentials; however, further structural and biochemical analysis described in the following sections precluded the need for further functional characterization of this channel. Although the functional changes elicited by the paddle swap are interesting unto themselves (the channel gating actually more resembles that of KvAP than hHv1), my major focus was to solve the structure of a Hv channel and, thus, only confirmation that the HAP5 chimera was a functional voltage-gated H⁺ channel was needed. The data presented in Fig. 4.14 and 4.15 demonstrate that the HAP5 chimera is functional.
Fig. 4.15 HAP5 is a voltage-gated H\(^+\) channel

(A) Whole-cell patch clamp recording from a HEK cell expressing hH\(_{V1}\). Voltage-step protocol was as indicated in the schematic. (B) Whole-cell patch clamp recording from a HEK cell expressing HAP5. Voltage-step protocol was as indicated in the schematic.

4.2.4 Structure of the HAP5\(\Delta N\Delta C\) chimera

Using the separate constant \((F_C)\) and variable regions \((F_V)\) of the Fab as search models for molecular replacement, it was possible to phase the low-resolution anisotropic data set. The unit cell (which was the asymmetric unit, since the space group was P1) was large enough to fit eight HAP5\(\Delta N\Delta C/33H1\) complexes; however, clear density was observed for only five and a half Fabs (6 \(F_V\)s and 5 \(F_C\)s), with the remainder of the cell being disordered. From the initial molecular replacement solution, tubular density was observed extending from the 33H1 binding sites, which unambiguously corresponded to the four transmembrane helices of the HAP5\(\Delta N\Delta C\) channel (Fig. 4.16).
Fig. 4.16 Example of helical electron density observed in a 33H1 Fab binding site

Stereo image of $1\sigma$ electron density from 2Fo-Fc map calculated from 33H1 Fab molecular replacement solution prior to any refinement or model building. Strands corresponding to 33H1 binding site are shown as green ribbons. Map was generated from data set summarized in Table 4.2 at 4 Å.

It was possible to fit the transmembrane helices into the density without any refinement of the initial search model (Fig. 4.17). Due to the low resolution and internal disorder of the data set, it was not possible to unambiguously determine the connectivity between the different transmembrane helices. However, due to the known interaction between the $K_v$AP paddle sequence and the 33H1 Fab (Jiang et al. 2003a), it was possible to identify and register the S3 and S4 helices. Additionally, it was clear from the density that the channels were arranged as dimers.

Comparing the arrangement of the four transmembrane helices of each HAP5ΔNΔC subunit it was evident that the observed structure was very different from that of known VSDs (Jiang et al. 2003a; Long et al. 2005; Long et al. 2007; Payandeh et al. 2011; Payandeh et al. 2012; Zhang et al. 2012). Additionally, the dimer interface in the structure was extensive, with the S3 helices wrapping around each other and
intercalating between the S3 and S4 helices of the opposite subunit in the dimer, causing a splaying of the paddle motif when compared to the KᵥAP structure (Fig. 4.18). The presence of this dimer interface along S3 disagreed with our site directed cross-linking studies of the wild type hHᵥ1 in membranes, which demonstrated that the dimer interface was along the S1 helix (see Chapter 2 and Lee et al. 2008).

In spite of these discrepancies seen in the model of HAP5ΔNΔC (Fig. 4.17) and the cross-linking data presented in Chapter 2, we knew from the electrophysiology and the flux assay of the reconstituted channel that the chimera was functional. However, the inconsistency between structural and biochemical data had to be resolved before further interpretation and refinement of the structure could be carried out.

Fig. 4.17 Helical model of HAP5ΔNΔC
Model of transmembrane helices generated by fitting polyalanine α-helices into the electron density found at the 33H1 binding site. Left, side view looking from within the expected membrane plane, right, top view looking down from the hypothetical extracellular side. The amino acid residues that corresponds to KᵥAP sequence are colored green, whereas hHᵥ1 derived residues are magenta.
Fig. 4.18 Comparison of paddle structures between HAP5 and K,AP isolated VSD
(A) Structural model of the HAP5ΔNΔC paddle colored as in Fig. 4.17. (B) Structural model of K,AP paddle from (Jiang et al. 2003a). (C) Overlay of paddle structures demonstrating that the paddle motif in HAP5ΔNΔC is splayed open compared to the equivalent sequence in K,AP. Also no kink is observed in the S3 helix of HAP5ΔNΔC.

4.2.5 Cross-linking indicates HAP5ΔNΔC chimera structure is not native

To probe the functional dimeric state of the HAP5 chimera, the channel was expressed in HEK cells in both full-length and C-terminally truncated forms and the pattern of site specific cross-linking was investigated and compared side-by-side with wild type hHv1 channels. In addition to the positions along S1 that were shown to form robust disulfide cross-links in hHv1, the amino acid residue positions along S3 that form the dimer interface in the structure (Fig. 4.17) were also mutated to cysteine one at a time and probed for cross-linking.
In both cases, the full-length and C-terminally truncated HAP5 chimera showed robust spontaneous disulfide bond formation along the top of the S1 helix, exactly recapitulating the pattern seen for wild-type hH\(_{\nu}\)1 (Fig. 4.19C). Because of the lack of the C-terminus from the truncated channels, the less specific anti-K\(_{\nu}\)AP antibody 6E1 was used in the western blots, which resulted in some non-specific signal in spite of which clear dimer was observed (Fig. 4.19E and G). Addition of oxidizing reagents and cysteine reactive cross-linkers to the membranes pushed cross-linking at these S1 sites to completion, just as was seen for the wild type hH\(_{\nu}\)1 channel (Fig. 4.19G; Chapter 2; Lee et al. 2008).

When the positions along S3 were probed for spontaneous cross-link formation, no disulfide bond formation was observed (Fig. 4.19B and F). Furthermore, addition of oxidizing reagents and cysteine-reactive cross-linkers still did not result in the observation of any cross-linked dimer (Fig. 4.19D and H). These data clearly indicated that the HAP5 chimera is a dimer in membranes even in the absence of the C-terminus, and that its dimer interface is along the S1 helix (i.e. similar to wild type hH\(_{\nu}\)1), not along the S3 helix as seen in the structure.
Fig. 4.19 Site-specific cross-linking studies of HAP5 and HAP5ΔC in membranes

(A) Cross-linking of the native coiled-coil cysteine (position 249) in both wild type hH\textsubscript{V}1 and HAP5. Production of a cys-less (CL) construct in which the two native cysteines have been mutated to serine precludes disulfide-mediated cross-linking. (B) Cysteine mutants of hH\textsubscript{V}1 CL along the S3 dimer interface seen in the crystal structure of HAP5ΔNΔC (C) Cysteine mutants along the S1-S2 loop dimer interface seen in the cross-linking studies discussed in Chapter 2 for both hH\textsubscript{V}1 CL and HAP5 CL constructs (D) Same S3 mutants of hH\textsubscript{V}1 CL as in (B) but under forcing conditions. (E) Comparing the effect of C-terminal truncation on the S1-S2 loop cysteine mediated cross-linking of HAP5 CL and HAP5ΔC CL (F) Cysteine mutants of HAP5ΔC CL along the S3 dimer interface seen in the crystal structure (G) Same S1-S2 mutants of HAP5 and HAP5ΔC CL as in (E) but under forcing conditions. (H) Same S3 mutants of HAP5ΔC CL as in (F) but under forcing conditions. Symbols in blue on the right on gels indicate the approximate positions for the dimer and monomer of the coiled-coil containing constructs (circles with tails) and the truncated constructs (circles without tails). (A – D) Use the αhH\textsubscript{V}1-coiled-coil antibody 9C1 as the primary antibody (E – H) Use the αK\textsubscript{V}AP-paddle antibody 6E1 as the primary antibody.
4.2.6 Final attempts at chimera strategy

Here, I will briefly describe my efforts to use the non-native HAP5ΔNΔC structure to design improved chimera with the goal of solving a native structure of the channel. The main question that needed to be addressed was whether the HAP5ΔNΔC was completely disordered in detergent or whether Fab binding was forcing the conformational equilibrium of the chimera into a low occupancy non-native conformation.

Since the Fab was not raised against the HAP5ΔNΔC channel itself but against the KvAP channel, if the paddle epitope were not properly presented for Fab recognition in the native chimeric construct, we would expect very little binding of the Fab to the native chimera. Therefore, four scenarios can be envisioned for binding of a Fab to an epitope-swapped chimeric channel: 1) the chimera structure is native and highly stable in detergent but does not present the epitope properly. In this case, Fab would not bind and we would see no complex on the SEC column. This may be true even if we saw binding of antibody in ELISAs and Western blots, since under these conditions we expect a higher proportion of unfolded channels. 2) The chimera is native and stable in detergent but does not present the epitope properly; however, it is in equilibrium with a low occupancy non-native (partially unfolded) state in which the Fab is able to bind to the paddle epitope with high affinity. In this case, we may see some incomplete complex formation on the SEC column, but high concentrations of Fab would result in the accumulation of non-native-chimera-Fab complex, which may then crystallize due to the strength of Fab mediated crystal contacts. 3) Although the native chimera doesn’t present the paddle epitope properly, the chimera is unstable in detergent and therefore significantly occupies a non-native conformation that is able to bind Fab with high
affinity. In this case, we would expect the chimera to be unstable in most non-dispersive detergents but bind strongly to the Fab resulting in complete complex formation on the SEC column. 4) The native chimera is highly stable in detergent and properly presents the paddle epitope in a way that is easily accessible to the Fab. In this case we would expect a biochemically stable construct and complete complex formation on the SEC column.

To solve the native structure of a chimeric channel we require chimera that fall into scenario 4 above. However, based on the evidence presented in this chapter, I conclude that HAP5ΔNΔC falls into scenario 2: it is biochemically stable in many detergents and it only forms partial complex with Fab on the SEC column. Therefore, the 33H1 Fab is only binding to a small population of non-native channels on the SEC column, but the structure is completely non-native when solved in complex with the 33H1 Fab. In addition, I categorize the nHAP3ΔC chimera into scenario 3: it is unstable in most detergents, but forms complete complex with Fab on the SEC column. The question then becomes: can we use what we see in the structure to build a chimera that falls into scenario 4, i.e. one that is stable in detergent and binds to the Fab in the native conformation?

By examining the structure, it was clear that the S1 and S2 helices were directly adjacent to the Fab; moreover, the S3-S4 helix-turn-helix paddle motif may be buried below these two adjacent helices in the native structure (Fig. 4.20A-C). If the paddle motif were buried, then Fab binding to the native structure would be prevented by steric clash between the Fab and the S1 and S2 helices.
Fig. 4.20 Structure based strategy for design of new chimera

(A) Cartoon representation of $K_v$AP VSD in complex with 33H1 Fab structure showing that the S3-S4 paddle epitope is extended relative to the S1-S2 loop. (B) Cartoon representation of HAP5ΔNΔC dimer indicating possible source of steric clash between the S1 and S2 helices and 33H1 Fab. (C) Schematic demonstrating how steric clash could lead to helix splaying. (D) Sequence of hH$_{v}1$ paddle; underlined portions indicates residues that were added to the paddle extension chimera. (E) Sequences of paddle extension chimera. (F) Schematic demonstrating how Fab binding could lead to disruption of helical packing (G) Sequences of alternate helical register chimera.
Looking back over the sequences of all the chimera constructed (Fig. 4.3), it was determined that, because of the register used to splice in the paddle sequence, the HAP5C construct actually contained the most extended paddle (the longest combined S3-S4 K\_\text{\textscript{\textit{V}}}AP/hH\_\text{\textscript{\textit{V}}}1 sequence) of all the chimeras originally screened. Therefore, four additional doubly truncated chimeras that shared the same register as HAP5 but with longer S3 and S4 helices were generated (Fig. 4.20E; nHAP8, nHAP10, nHAP11 and nHAP12 were constructed). These chimera were expressed, purified and tested for only the two scenario-4 properties: biochemical stability and complete complex formation with Fab on the SEC column.

With the exception of nHAP10\DeltaN\DeltaC (which was approximately one helical turn longer on S3 and S4 with respect to HAP5), most of the paddle extensions resulted in decreased stability of the chimeric constructs. Although the nHAP10\DeltaN\DeltaC chimera was stable, the alterations did not improve Fab binding. The binding of nHAP10\DeltaN\DeltaC to both 6E1 and 33H1 Fabs looked very similar to that of HAP5\DeltaN\DeltaC, with only a small proportion of complex formation, likely placing it into scenario 2.

Another possible way in which the paddle epitope may be incorrectly presented for Fab binding on the chimeric channel is if the register of the S3 and S4 helices is wrong relative to that of native K\_\text{\textscript{\textit{V}}}AP. If S3 is longer or shorter than S4 in the chimera relative to the equivalent positions of the K\_\text{\textscript{\textit{V}}}AP S3 and S4 helices, then the S3-S4 helix turn helix motif may be askew, resulting in poor Fab binding and disruption on helical packing when the Fab does bind (Fig. 4.20F). To address this, eight additional chimeric constructs were built: four in which successive single amino acid residue deletions were carried out in S3 and four in which successive single amino acid residue additions were
carried out in S4 (Fig. 4.20G). These deletions/additions were carried out on the background of the nHAP10ΔNΔC construct, since it was the most stable chimera with the most extended paddle motif. Expression and purification of these eight constructs demonstrated that the nHAP10ΔNΔC S3-1, S3-1 and S4+4 constructs (Fig. 4.20G) were very stable and monodisperse on the sizing column. However, none of these constructs improve binding of either 6E1 of 33H1 Fabs.

Although there are a great number of possible combinations of amino acid residue additions/deletions that could be tested for these paddle chimeras, after failure of this set of eight constructs it was decided that it would be more productive to pursue other avenues for improved Fab binding. Therefore, the paddle chimera strategy was abandoned in favor of raising Fabs against the hH vbΔNΔC channel by phage display (discussed below) and of nuclear magnetic resonance (NMR) characterization of the wild type channel (discussed in the next chapter).

4.3 RAISING FABS BY PHAGE DISPLAY

4.3.1 Phage display selections overview

For soluble proteins, phage display selections are done via biotinylation and immobilization of the protein of interest onto streptavidin or neutravidin coated 96-well plates (Sidhu et al. 2000). In this way, large phage libraries with >10^{13} sequence diversity can be applied to the sample for selection of binders by successive washing steps (Persson et al. 2013). In practice, the phage libraries are applied first to a streptavidin-coated well alone to remove any phage clones with non-specific or streptavidin specific
binding, and then the phage are moved to the wells with the immobilized sample (Sidhu et al. 2000). After incubation and 4-5 rounds of washing, the bound phage are eluted from the sample by addition of acid and are then amplified overnight in bacterial culture. This process is repeated in four or five rounds of selection, using higher concentrations of phage in each successive round. Increasing the phage concentration as the selection rounds progress results in greater competition between the remaining phage, selecting for the higher affinity binders (Sidhu et al. 2000). After the final round of selection, phage-infected bacteria are plated out in order to isolate single clones, which are then expanded and tested for binding to the antigen by ELISAs. Positive-binding clones are then sequenced and the Fabs present can be easily cloned, expressed and purified for use in structural studies (Sidhu et al. 2000).

This process was very much the same for purified membrane proteins, except that each step required the presence of detergents to keep the proteins in solutions. Due to the long time course of the selection process (generally 4-5 days), it is necessary that the membrane protein of interest be highly stable in detergent over long time spans. For most membrane proteins, including hH\(_V\)1, this is not the case, complicating the phage-display selection process. Nonetheless, because of our desire for high-affinity antibodies against the hH\(_V\)1 transmembrane domain, we initiated a collaboration with the laboratory of Dr Sachdev Sidhu at the University of Toronto. Before going to Dr Sidhu’s laboratory, I was able to show that the hH\(_V\)\(\Delta N\Delta C\) construct was efficiently biotinylated using two different reagents: one that reacts specifically with cysteine residues and one that reacts with primary amines such as the side chains of lysine residues.
Working with postdoctoral fellow Dr Yuko Arita, I carried out five rounds of phage selections against the hH$_V$ΔNΔC channel in diheptanoyl-glycerol-phosphatidylcholine (DHPC) detergent using two different phage-Fab libraries (Fellouse et al. 2007; Persson et al. 2013). After several days of selections, it was clear that the channel was becoming unstable, as small amounts of precipitate were seen in the sample tubes. Although we were able to prevent the protein from precipitating by diluting our stock concentration, we were still unsuccessful in isolating any strong binders from our selections. Instead, we obtained a plethora of phage that bound non-specifically to the plates and did not express functional Fab domains: PCR and sequencing analysis indicated either the presence of no Fab, or of frame-shift or nonsense mutations within the complementarity determining regions (CDRs).

An abundance of non-specific “sticky” binders is common where there are no strong specific binders present in the phage library, or if there is heterogeneity in the target sample such that the selection pressure for strong binders is low. Because of the long time course required for the selections and the inherent instability of hH$_V$ΔNΔC channel in detergent, we concluded that it would be unproductive to continue attempting selections according to this standard protocol. However, due to my experience with channel reconstitution and functional analysis (see Chapter 3) it occurred to me that it would be beneficial to develop a phage-selection protocol using reconstituted channels.

The hH$_V$ΔNΔC channels in vesicles are highly stable, and reconstituted vesicles can be frozen and stored at -80°C until needed. By using the reconstituted channels, the buffers needed for selections and ELISAs would no longer require detergents, thereby greatly reducing the cost of the entire protocol. Efficient negative selections could be
carried out using vesicles that did not contain any hH\textsubscript{v}ΔNΔC channels, thereby removing any phage that non-specifically bound to lipids. Furthermore, the activity of the reconstituted hH\textsubscript{v}ΔNΔC channels could be tested by the fluorescence-based concentrative uptake flux assay described in Chapter 3, thus allowing confirmation that the channels were in a functional native state. Therefore, we decided to develop this new phage-display library selection method for reconstituted membrane proteins.

4.3.2 Vesicle-based selections of reconstituted hH\textsubscript{v}ΔNΔC

Another benefit of using the vesicle-based selections is that it is possible to incorporate biotin-modified lipids into the membrane, thereby bypassing the need for potentially structure-altering modifications (biotinylation) of the protein of interest. In the case of the hH\textsubscript{v}ΔNΔC, vesicles reconstitution was done in 3:1 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE): 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG) lipids doped with 0.5% 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) headgroup modified PE (DPPE-biotin). Assuming an average vesicle radius of 100 nm, this concentration of DPPE-biotin would result in approximately 30-40 DPPE-biotin molecules per vesicle or 15-20 DPPE-biotin per membrane leaflet. Vesicles prepared in this way should be efficiently captured by streptavidin-coated plates.

To test streptavidin binding to the reconstituted DPPE-biotin containing vesicles, sucrose flotation assays were carried out on a sample of the vesicles to determine whether the streptavidin would migrate with the vesicles in the sucrose gradient. In the presence of streptavidin, the DPPE-biotin containing vesicles formed a gel at the interface of the
10% and 30% sucrose solutions in the step gradient. This gel phase was not observed with the DPPE-biotin containing vesicles in the absence of streptavidin or with normal vesicles not containing DPPE-biotin in the presence of streptavidin. Since streptavidin is a tetramer in solution, the gel phase is likely formed by cross-linking from the binding of multiple DPPE-biotin-containing vesicles by single streptavidin tetramers. These observations led me to conclude that the DPPE-biotin was presented on the surface of the vesicles in a way that allows for efficient streptavidin capture of the biotin-modified headgroup.

For the selections, the hHvΔNΔC were reconstituted into the DPPE-biotin containing vesicles at a protein-to-lipid ratio of 1:100 (wt:wt). At this ratio, there should be approximately 20 channels per vesicle on average. To confirm efficient incorporation of the channel into the vesicles, a sucrose flotation assay was performed and the functional state of the channels was tested with the fluorescence-based flux assay. These data showed that the hHvΔNΔC channels were efficiently incorporated into the vesicles and that the reconstituted channels were functional (Fig. 4.21).
Fig. 4.21 H⁺ uptake into vesicles containing truncated hHv.1 channels

Fluorescence-based H⁺ flux assay for vesicles containing hHvΔNΔC (purple) compared to empty vesicles (orange) and wild type hHv.1 containing vesicles (cyan). Valinomycin and CCCP were added at the indicated time points and the fluorescence measurements were repeated 3-4 times (error bars represent the standard error of the mean).

When Dr Arita performed the phage selections with DPPE-biotin-containing vesicles, the use of empty vesicles for negative selection and of hHvΔNΔC-containing vesicles for positive selections markedly improved the results compared to those of the detergent-based selections. As opposed to the detergent-based selections (which did not produce any potential Hv binding Fab clones), the vesicle-based selections generated nine Fab clones that showed significant selective binding to hHvΔNΔC-containing vesicles (Table 4.3). Dr Arita subcloned these Fab genes into an expression vector and I expressed and purified each of the Fabs with the exception of Fab9 (Table 4.3), which did not express.
Table 4.3 ELISA Data for the best Fabs isolated from vesicle based selections

<table>
<thead>
<tr>
<th>Fab ID</th>
<th>BSA</th>
<th>Empty</th>
<th>FLAG</th>
<th>hHᵩΔNΔC</th>
<th>FLAG/BSA</th>
<th>hHᵩΔNΔC/Empty</th>
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<tr>
<td>Fab2</td>
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<td>0.839</td>
<td>1.236</td>
<td>10.2</td>
<td>4.3</td>
</tr>
<tr>
<td>Fab3</td>
<td>0.086</td>
<td>0.434</td>
<td>0.116</td>
<td>1.664</td>
<td>1.3</td>
<td>3.8</td>
</tr>
<tr>
<td>Fab4</td>
<td>0.100</td>
<td>0.164</td>
<td>0.996</td>
<td>1.433</td>
<td>10.0</td>
<td>8.7</td>
</tr>
<tr>
<td>Fab5</td>
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<td>1.065</td>
<td>0.647</td>
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<td>3.4</td>
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<tr>
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<td>0.856</td>
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<td>0.114</td>
<td>0.695</td>
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<td>3.1</td>
</tr>
</tbody>
</table>

These ELISAs were performed by Dr Yuko Arita

‡ Raw ELISA data corresponds to wells containing BSA control (BSA), Empty Vesicles (Empty), hHᵩΔNΔC containing vesicles probed with an αFLAG Ab (FLAG), hHᵩΔNΔC containing vesicles. All wells were probed with αM13 phage Ab unless otherwise stated. The Fab gene on the phage contains a C-terminal FLAG-tag therefore the αFLAG antibody will only bind to phage that expresses a full-length Fab gene.

* High FLAG/BSA ratio indicates the presence of a bound phage that expresses a full-length Fab gene. High hHᵩΔNΔC/Empty ratio indicates specific phage binding to hHᵩΔNΔC. The two best hHᵩΔNΔC/Empty ratios are highlighted in green.

The remaining eight Fabs were tested for binding to the hHᵩΔNΔC channel in DHPC. Only Fab2 showed evidence of weak binding to the channel on the SEC column in detergent (Fig. 4.22). Although there was almost no complex seen on the SEC column, a shift to lower elution volumes of some Fab2 was observed when the fractions were run on an SDS-PAGE gel (Fig. 4.22C). This shifting of elution volume was not observed for the other best candidate, Fab4 (Table 4.3), indicating that Fab2 did bind weakly to the channel in DHPC. However, the binding observed for Fab2 was very weak and was not considered significant enough to carry forward into full-scale crystallization trials.
Fig. 4.22 Evidence for weak complex formation between Fab2 and hHvΔNΔC
(A) SEC chromatogram of hHvΔNΔC with Fab2. (B) SEC chromatogram of hHvΔNΔC with Fab4. (C) SDS-PAGE reducing gel of SEC fractions showing a shift in the elution volume for Fab2 indicating weak binding of Fab2 to hHvΔNΔC.

4.3.3 Future directions

Although Fab2 showed some evidence of weak binding, stronger-binding Fabs are needed to justify full-scale crystallization trials. Therefore, Dr Arita is developing affinity-matured libraries based on the sequences of the best binders from the vesicle-based selections (Fab2 and Fab4; Table 4.3). These libraries are currently being screened for Fabs with improved binding to vesicle-reconstituted hHvΔNΔC. If Fabs with a significant improvement in binding are identified, they will be tested for Fab-channel complex formation by SEC. All promising candidates will be followed up in crystallization trials.
4.4 Conclusions

In this chapter, I described my efforts to obtain the X-ray crystal structure of a H\textsubscript{v} channel. Together with Dr Seok-Yong Lee we attempted crystallization of the wild type channel, lysozyme and dimer fusion constructs, and chimeric channels. Although I did obtain a crystal structure with the HAP5ΔNΔC chimera, I concluded that this structure was non-native. Because of the power of Fab mediated crystallization it is still desirable to isolate high affinity antibodies against the transmembrane domain of hH\textsubscript{v}1. My work in collaboration with Dr Yuko Arita in the Sidhu lab at the University of Toronto on the isolation of phage display Fabs has the potential to achieve this goal. However, if we are unable to isolate high affinity antibodies by this method it may still be possible to get high affinity crystallization chaperones by other means. By injecting hH\textsubscript{v}1 into llamas it may be possible to generate hH\textsubscript{v}1 specific nanobodies. These single-chain camelid antibody fragments have been used successfully for the crystallization of membrane proteins that lack well-structured soluble domains (Rasmussen et al. 2010).

As an alternative strategy for structure determination, working with Dr Joel Butterwick in the laboratory, I decided to pursue NMR characterization of hHV1ΔNΔC. As described in the next chapter, this methodology allowed us to define the secondary structure of the channel and we experimentally identified a short N-terminal S0 helix that precedes the transmembrane domain.
CHAPTER 5: NMR

During the final stages of the crystallography work discussed in Chapter 4, I set out to also study the chimeric HAP5ΔC by nuclear magnetic resonance (NMR) with detergent-solubilized channels. Together with postdoctoral fellow Dr Joel Butterwick, I planned to examine the titratable amino acid residues within the transmembrane domain of the channel in order to investigate the conduction mechanism. Because of the sensitivity of NMR to the protonation state of amino acid side chains, this technique complements the crystallographic studies and can provide additional insight into the mechanism of H⁺ conduction through Hᵥ.

Although our studies began with the chimeric HAP5ΔC channels, after the crystallography indicated that this channel might not be properly structured in detergent, we decided to work on the wild type truncated hHᵥΔNΔC channel. In this chapter, I will briefly discuss our initial NMR work on the HAP5ΔC channel and will then present our efforts to solve the hHᵥΔNΔC channel structure.
5.1 NMR ON THE HAP5 CHIMERA

5.1.1 Removal of N-terminus allows for detection of transmembrane peaks

The HAP5ΔC construct was chosen as the best candidate for NMR because of its stability at high concentrations at room temperature (required to maintain its homogeneity during the multi-day length of the experiments) and because of the progress that its structural characterization by X-ray crystallography showed at the time.

To collect a $^1$H-$^{15}$N heteronuclear single quantum coherence (HSQC) spectrum, the protein had to be uniformly labeled with the heavy $^{15}$N isotope. Since the only source of nitrogen during the expression of the channel in Pichia pastoris was from the ammonium sulfate added to the media, we were able to uniformly label the protein by replacing all of the ammonium sulfate with $^{15}$N-ammonium sulfate.

The original spectra collected on the HAP5ΔC in LDAO channel showed many peaks (Fig. 5.1). Since every peak on the $^1$H-$^{15}$N HSQC spectrum corresponds to a covalently bound $^1$H-$^{15}$N pair, the majority of the peaks seen derived from the main chain amide bond, with a subset originating from the side chains of asparagine, glutamine and tryptophan residues (Cavanagh et al. 2007). Depending on the local environment of the amino acid residue, it is sometimes possible to see peaks corresponding to the side chains of arginine residues, but it is very rare to see peaks corresponding to histidine and lysine side chains because of their fast rate of H$^+$ exchange with the solvent (Cavanagh et al. 2007).
Fig. 5.1 $^1$H-$^{15}$N HSQC spectrum of $^{15}$N labeled HAP5ΔC and tHAP5ΔC in LDAO

(A) $^1$H-$^{15}$N HSQC spectrum of HAP5ΔC in N,N-Dimethyldodecylamine N-oxide (LDAO) at 25°C (top) with the glycine resonances highlighted to demonstrate the eclipsing effect of the N-terminus. Four glycine resonances, which likely correspond to the four glycines in the N-terminus are much stronger than two other glycine peaks observed that likely correspond to glycines from the transmembrane segment. (B) $^1$H-$^{15}$N HSQC spectrum of trypsinized HAP5ΔC (tHAP5ΔC) in LDAO at 25°C (bottom) demonstrating that the majority of the intense peaks derived from the N-terminus.
The intensity of the peaks observed on the $^{1}\text{H}$$^{15}\text{N}$ HSQC is positively correlated to the flexibility of the protein; thus, loops and unstructured regions might eclipse the more stable parts of the protein (Cavanagh et al. 2007). This can be worse with detergent-solubilized membrane proteins since the interactions with the detergent micelle can further broaden peaks corresponding to the transmembrane domain (Butterwick & Mackinnon 2010). Therefore, we suspected that the strong peaks observed in the $^{1}\text{H}$$^{15}\text{N}$ HSQC spectrum of the HAP5ΔC channel originated from residues within the unstructured N-terminus (Fig. 5.1A). For example six peaks corresponding to glycine resonances can be seen in the $^{1}\text{H}$$^{15}\text{N}$ HSQC spectrum of the HAP5ΔC (Fig. 5.1A) the four intense glycine peaks likely correspond to the four glycines found in the N-terminus, whereas the two weaker glycine peaks likely correspond to glycines from the transmembrane domain (Fig. 5.1A).

To isolate the peaks originating from the transmembrane domain, we collected a spectrum from trypsinized HAP5ΔC (tHAP5ΔC) channels in which the digested N-terminus had been purified away (Fig. 5.1B). This spectrum clearly indicated that the unstructured N-terminus was indeed eclipsing the transmembrane region in the spectrum of the untrypsinized HAP5ΔC and that, if we wanted to examine the titratable residues in the transmembrane domain, we would have to work with the doubly truncated HAP5ΔNΔC construct.

To improve to resolution of the spectrum, it was desirable to perdeuterate the protein. By using transverse relaxation-optimized spectroscopy (TROSY), it is possible to sharpen the line-width of the peaks, making it easier to make out individual $^{1}\text{H}$$^{15}\text{N}$ peaks (Cavanagh et al. 2007). Although the expression levels were greatly reduced by growing
the *Pichia pastoris* in heavy water (\(^2\)H\(_2\)O a.k.a. D\(_2\)O), we were able to purify a sample of perdeuterated HAP5\(\Delta\)N\(\Delta\)C. By perdeuteration and by changing detergents from LDAO to the lipid-like detergent 1-palmitoyl-2-hydroxy-sn-glycero-3-phospho-(1'-rac-glycerol) (LPPG) the resolution of this spectrum was greatly improved (Fig. 5.2).
Fig. 5.2 Spectra of HAP5ΔNΔC in LPPG

(A) $^1$H-$^{15}$N HSQC spectrum of $^{15}$N-labeled HAP5ΔNΔC in LPPG at 25°C. Peaks that are aliased in the $^{15}$N dimension are shown in black. All five expected glycines can be seen, as well as the side chains of the arginine, asparagine, glutamine and tryptophan (inset).
residues. (B) $^1$H-$^{15}$N HSQC TROSY of $^2$H-$^{15}$N labeled HAP5ΔNΔC in LPPG at 25°C. Peaks that are aliased in the $^{15}$N dimension are shown in blue.

5.1.2 Abandonment of the chimeric channel in favor of wild type

With spectra of perdeuterated, $^{15}$N-labeled HAP5ΔNΔC in our hands, we planned to begin the assignment of the $^1$H-$^{15}$N HSQC spectrum peaks. However, at this time, the results from the crystallography experiments indicated that, although the HAP5 chimera is a functional H$^+$ channel, its structure might be destabilized by extraction from the membrane and solubilization by detergent (see Chapter 4). Therefore we decided that it would be best not to pursue further NMR analysis of the HAP5ΔNΔC chimeric construct.

However, my work with the chimeric proteins, discussed in Chapter 4, required the redesign of the protein expression construct, which resulted in greatly improved yields after purification. We reasoned that if we were to apply the same changes to the wild type hH$_\nu$ΔNΔC construct (i.e. moving the GFP tag and PreScission protease site to the N-terminus), we should also improve the yield of purified wild type channel. In addition, if detergent conditions could be found in which this channel was highly stable it should be possible to examine the wild type hH$_\nu$ transmembrane domain by NMR.

Dr. Butterwick had previously solved the structure of the isolated voltage-sensor domain (VSD) of K$_\nu$AP by NMR (Butterwick & Mackinnon 2010). Therefore, although we were unable thus far to solve the structure of the hH$_\nu$ΔNΔC construct by crystallography (see Chapter 4), we reasoned that with improved expression and stability it might be possible to solve the structure by NMR.
5.2 NMR STUDIES OF HUMAN H\textsubscript{\textnu} CHANNELS

5.2.1 Truncated hH\textsubscript{\textnu}1 channels are functional

In addition to the functional analysis of the reconstituted hH\textsubscript{\textnu}ΔNΔC channels by the flux assay presented in Chapter 4 (Fig. 4.21 on page 133), electrophysiological studies were carried out on the truncated versions of the hH\textsubscript{\textnu}1 channel. In order to confirm that the hH\textsubscript{\textnu}ΔNΔC channel construct used for NMR studies was a functional H\textsuperscript{+}-selective voltage- and ΔpH-gated channel, whole-cell patch clamp electrophysiology of truncated channels over-expressed in HEK cells was performed. Due to the importance of the ΔpH gating in the physiological functions of the channel and its poorly understood molecular mechanism (see Chapter 1), we wanted to confirm that any construct that we pursued for structural analysis maintained this important mode of regulation. For completeness, all three truncated channel constructs (hH\textsubscript{\textnu}ΔN, hH\textsubscript{\textnu}ΔC and hH\textsubscript{\textnu}ΔNΔC) were characterized and compared to wild type.

The electrophysiological recordings clearly indicated that, although the truncations altered the gating kinetics, all of the truncated channels were functional voltage-gated channels (Fig. 5.3). Furthermore, by perfusion of the external solution with buffers at different pH, it was possible to both measure the ΔpH gating and H\textsuperscript{+} selectivity of the channels (Fig. 5.3). These results indicated that, although the truncations did alter ΔpH-gating behavior, the truncated channels were still ΔpH-gated (Fig. 5.3). By comparing the reversal potentials of the measured currents to what is expected for the Nernst equilibrium potential for H\textsuperscript{+} at the different ΔpHs, it was possible to show that each of the constructs maintained wild-type-like H\textsuperscript{+} selectivity (Fig. 5.4).
Fig. 5.3 Electrophysiological characterization of truncated hHv1 channels
Examples of currents elicited from whole-cell patch clamp recording of HEK cells expressing (A) hHv1, (B) hHvΔN, (C) hHvΔC and (D) hHvΔNΔC at symmetric pH (pH_o = pH_i = 6.5; left) according to the voltage-step protocols shown. Average tail current IV curves corresponding to the same cell as shown on the right at three different pH_o (pH_o = 7.0 blue, pH_o = 6.5 brown and pH_o = 6.0 green). Each recording was performed 2-3 times per cell per pH_o with the error bars on the IV curves corresponding to the standard deviation are plotted but are smaller than the graph symbols in most cases.
Fig. 5.4 H⁺ selectivity of wild type and truncated hHv1 channels

Examples of currents elicited from whole-cell patch clamp recordings of HEK cells expressing hHvΔNΔC at (A) pHₒ = 6.0 (B) pHₒ = 6.5 and (C) pHₒ = 7.0 according to the voltage-step protocols shown. In each case pHᵢ = 6.5. (D) IV plot of average current elicited shortly after variable voltage-step for hHv1 (cyan), hHvΔN (red), hHvΔC (green) and hHvΔNΔC (purple) according to the same voltage-step protocols show in (A-C) at the different values of pHₒ. Each recording was performed 3-4 times per cell per pHₒ, error bars corresponding to the standard deviation are plotted but are smaller than the graph symbols in most cases. (E) Comparison of the reversal potentials for each construct (colored as in D) to the Nernst equilibrium potential for H⁺ (black line) at each pHₒ. Each point represents the average of 3-4 cells with the error bars corresponding to the standard deviation.
It is interesting to note the opposite effect that the N- and C-terminal truncations have on the gating kinetics and ΔpH-gating. The N-terminal truncation decreased the rate of both opening and closing of the channel (Fig. 5.3B). Additionally, truncation of the N-terminus also shifted the ΔpH-gating of the channel, resulting in channel opening at left-shifted (more negative) voltages relative to wild type channels at the equivalent ΔpH (Fig. 5.3B). Unlike the wild type channel, in which ΔpH-gating ensures that the channel is a perfect outward rectifier (see Chapter 1), this left shift of the delta-N channel allows small inward H⁺ currents at high external H⁺ concentrations (low external pH). In contrast, the C-terminal truncation increases the rate of both opening and closing of the channel (Fig. 5.3C) and shifts the ΔpH-gating such that channel opening occurs at more right-shifted voltages, strengthening the wild-type-like outward rectification (Fig. 5.3A and C). Although similar to the C-terminal truncation alone in that the rates of channel opening and closing are sped up in the doubly truncated channel (hH₉ΔNΔC), the ΔpH-gating more resembles that of wild type channels (Fig. 3D).

These data support a potential interaction between the N-terminus and transmembrane domain in wild type channels. Such an interaction has been previously proposed by Musset et al., based on modulation of the gating of wild type hH₁ by phosphorylation of amino acid residue T29 on the N-terminus (Musset et al. 2010a). A simple explanation for the changes in channel gating observed upon N-terminal truncation or phosphorylation could be that the unphosphorylated N-terminus binds to the transmembrane domain and stabilizes the hyperpolarized conformation. If true, this would mean that disruption of the interaction between the N-terminus and the transmembrane domain by either removal or phosphorylation of the N-terminus would
destabilize the hyperpolarized conformation, resulting in the observed left-shift in the voltage activation of the channel (Fig. 5.3B; Musset et al. 2010a).

More research is required to fully understand the interactions between the termini and the transmembrane domain and to identify the amino acids residues involved. However, the data presented in Fig. 5.3 demonstrate that the truncated channels are functional voltage- and ΔpH-gated H⁺-selective channels, validating the hHvΔNΔC construct as a good structural target for generating insight into these interesting biophysical phenomena.

5.2.2 hHvΔNΔC is biochemically stable in two detergents

Expression of wild type hHvΔNΔC was much improved by the N-terminal decaHis-GFP tag. In order to get sufficiently enriched channel for the NMR experiments, it was also necessary to alter the purification protocol. Originally, the purification was carried out similar to that of the HAP5ΔNΔC construct in DM (discussed in Chapter 4), i.e. via Co²⁺-resin immobilized metal affinity chromatography (IMAC), followed by PreScission protease digestion, a second round of Co³⁺-resin IMAC for removal of the cleaved decaHis-GFP tag and a by final size exclusion chromatography (SEC). However, dialyzing into low salt during overnight PreScission protease digestion and replacing the second IMAC step with an anion exchange chromatography step were sufficient to significantly improve the purity of the hHvΔNΔC channel.

I investigated the detergent stability profile of the truncated channel of this highly enriched hHvΔNΔC. The hHvΔNΔC channel was unstable in maltoside detergents,
precipitating after one day at room temperature at high concentrations. However, the truncated channel was highly stable in the lipid-like detergents DHPC and LPPG. In either of these detergents, the truncated channel remained in solution and monodisperse on the SEC column at high concentrations after incubation for several days at room temperature.

During detergent exchange into LPPG, a slow timescale detergent-exchange phenomenon was observed, which manifested as a significant shift in the elution volume of the hH_vΔNΔC peak (Fig. 5.5). Specifically, after the ion exchange chromatography step in DM the channel was concentrated and run over the SEC column equilibrated in LPPG. During this detergent exchange SEC run the hH_vΔNΔC channel eluted as a broad peak centered at 12.8 mL (Fig. 5.5A). When this peak was pooled, concentrated and re-run over the SEC column in LPPG, the channel eluted as a doublet with peaks centered at 12.4 and 13.4 mL respectively (Fig. 5.5B). If the same sample was re-run over the SEC column after several hours, the elution profile collapsed into a single sharp peak centered at 13.3 mL, which then remained stable for up to a week at room temperature (Fig. 5.5C). Such a detergent-exchange phenomenon was not observed for exchange of hH_vΔNΔC from DM to DHPC.
Fig. 5.5 DM to LPPG detergent exchange phenomenon of hH$_v$$\Delta$$\Delta$NC

(A) SEC chromatogram of hH$_v$$\Delta$$\Delta$NC initial detergent exchange from DM to LPPG. (B) SEC chromatogram of immediate concentration and rerun of pooled fractions 10-14 mL from (A) over SEC column equilibrated in LPPG. (C) SEC chromatogram of rerun of pooled fractions 10-14 mL from (A) over SEC column equilibrated in LPPG after 1 day incubation at room temperature (RT) at 6 mg/mL.
Due to the large change observed when hH\textsubscript{v}\Delta N\Delta C was exchanged into LPPG, we were concerned that we were obtaining either incomplete detergent exchange or a detergent-dependent refolding of the channel, potentially into a non-native conformation. We were able to rule out incomplete detergent exchange as a possible cause by looking at the \textsuperscript{1}H peaks corresponding to the detergent in the NMR spectrum. Due to the high concentration of detergent in the concentrated NMR samples, the strongest peaks in the one-dimensional \textsuperscript{1}H spectrum come from the detergent molecules. If there were incomplete detergent exchange between DM and LPPG, we would expect to see \textsuperscript{1}H peaks corresponding to DM molecules in the spectrum. However, only peaks corresponding to LPPG were observed, indicating that complete detergent exchange had occurred. This is also in agreement with the observation that the exchange phenomenon occurs to the concentrated hH\textsubscript{v}\Delta N\Delta C samples in the absence of further detergent exchange (i.e. given time the sample will convert to the 13.3 mL peak without further runs over a SEC column equilibrated with LPPG).

When we collected a \textsuperscript{1}H-\textsuperscript{15}N HSQC spectrum of the hH\textsubscript{v}\Delta N\Delta C construct in LPPG, we observed a highly disperse set of peaks corresponding to the majority of the hH\textsubscript{v}\Delta N\Delta C main chain amides as well as all of the asparagine, glutamine, arginine and tryptophan side chains (Fig. 5.6A). Since the quality of the HSQC spectrum is sensitive to the dynamics of the protein, this high-quality spectrum indicated that the channel was in a well-folded stable confirmation (Cavanagh \textit{et al.} 2007). We concluded that the probability of the channel adopting a well-folded stable non-native conformation was low and that, therefore, the conformation of the channel after the detergent exchange phenomenon was likely native.
Fig. 5.6 Detergent screen $^1$H-$^{15}$N HSQC of $^{15}$N labeled hH$_{\Delta}$N$\Delta$C

(A) $^1$H-$^{15}$N HSQC spectrum of $^{15}$N-labeled hH$_{\Delta}$N$\Delta$C in LPPG. (B) $^1$H-$^{15}$N HSQC spectrum of $^{15}$N-labeled hH$_{\Delta}$N$\Delta$C in LDAO. (C) $^1$H-$^{15}$N HSQC spectrum of $^{15}$N-labeled hH$_{\Delta}$N$\Delta$C in DHPC. All spectra were collected at 25°C. Peaks that are aliased in the $^{15}$N dimension are shown in blue.
Although the hHvΔNΔC channel was less stable in LDAO than in LPPG or DHPC, we decided to examine the \(^1\text{H}-^{15}\text{N}\) HSQC spectrum in LDAO because of the high quality spectrum observed for the chimeric HAP5ΔNΔC in this detergent (Fig. 5.1B). However, the \(^1\text{H}-^{15}\text{N}\) HSQC spectrum of hHvΔNΔC in LDAO was very weak with only few distinguishable peaks (Fig. 5.6B).

Detergent exchange of hHvΔNΔC from DM to DHPC did not result in any equivalent slow exchange phenomenon. During initial detergent exchange into DHPC hHvΔNΔC elutes as a single peak centered at 14.8 mL and this peak position does not change significantly with time. However, when we collected a \(^1\text{H}-^{15}\text{N}\) HSQC spectrum in DHPC the quality of the spectrum was poor (Fig. 5.6C). The poor quality of the \(^1\text{H}-^{15}\text{N}\) HSQC spectrum in DHPC prohibited any further structural analysis of hHvΔNΔC by NMR in this detergent. Therefore, we concluded that structural work by NMR on hHvΔNΔC would be pursued in LPPG.

5.2.3 Assignment of the hHvΔNΔC spectra in LPPG

Resonance assignments for backbone (\(^1\text{H}^N\), \(^{15}\text{N}\), \(^{13}\text{C}'\), and \(^{13}\text{C}''\)) nuclei at 25°C and neutral pH were identified using TROSY HNCA, HNCO and HN(CO)CA. Moreover, \(^{15}\text{N}\)-edited \(^1\text{H}^1\text{H}\) nuclear Overhauser effect spectroscopy (NOESY) experiments (Cavanagh et al. 2007) were recorded using 70% deuterated hHvΔNΔC samples. Isotope labeling of the His-GFP-hHvΔNΔC construct was performed according to the same protocol used for the HAP5 constructs (see above and Materials and Methods), with the exception that the concentration of D\(_2\)O was lowered from 90% to 70%, greatly improving the expression
with no significant reduction in TROSY spectrum quality. Uniform $^{13}$C labeling of the hH$_v$$\Delta$N$\Delta$C construct was accomplished by replacing the glycerol in the growth media and methanol in the expression media with $^{13}$C-labeled glycerol and methanol respectively.

In addition to the uniformly labeled samples, we recorded HSQC experiments on samples with different combinations of labeled amino acids so that specific amino acids could be distinguished in crowded regions of the spectra: A, G, F, D/N, L, I, K, E/Q, V and R (for examples see Fig. 5.7A and B). Although bacterial expression had not resulted in any extractable channel for the original hH$_v$1 constructs (see Chapter 3), we were able to express and purify the His-GFP-hH$_v$$\Delta$N$\Delta$C construct from *Escherichia coli* which, due to significantly reduced metabolic shuffling of labeled atoms into other amino acids, facilitated specific amino acid labeling. Resonance assignments were extended along the side chains using H(C)CH-COSY and $^{13}$C-edited and $^{15}$N-edited NOESY experiments. Most ambiguities present among the methyl resonances were resolved by repeating the $^{13}$C-edited NOESY using methyl-specific labeling on Ile, Leu, and Val residues (Cavanagh *et al.* 2007). Complete backbone resonance assignments (H, N, CA, CO) were determined for 56% of the 138 residues, whereas 77% of residues have main chain amide assignments (Fig. 5.7). Importantly, peaks for 84% have at least some (main chain or side chain) assignments, providing easily accessible probes for nearly every residue within the hH$_v$$\Delta$N$\Delta$C construct.
Fig. 5.7 Amide resonance assignments for hH$_{\text{V}}$ΔNΔC in LPPG micelles

$^1$H-$^{15}$N HSQC spectra of (A) $^{15}$N-Ile/Gly ($^{15}$N-Ser is also visible because of metabolic scrambling of $^{15}$N-Gly label) and (B) $^{15}$N-Arg specific amino acid labeled H$_{\text{V}}$ΔNΔC in LPPG. (C) $^1$H–$^{15}$N HSQC TROSY spectrum at 25°C of $^2$H-$^{15}$N labeled hH$_{\text{V}}$ΔNΔC with assignments. Main-chain amide peaks for 106 residues (out of 136 residues expected to be observed; 2 Pro) and N132$^b$, N133$^b$, N214$^b$, Q102$^e$, Q128$^e$, Q191$^e$, Q194$^e$ and W207$^e$ side chains have been assigned. Peaks that are unassigned are shown in black. Peaks that are aliased in the $^{15}$N dimension include G90, G199, G215 and the Arg$^e$ resonances.
5.2.4 Secondary structure shows S1-S4 helices plus a S0 helix

Chemical-shift analysis of the assigned main chain resonances allowed for the determination of secondary structure of the hHvΔNΔC construct (Fig. 5.8). Patterns of secondary chemical shifts of main chain resonances ($\Delta \delta = \text{observed shift} - \text{“random coil” shift}$) report on the secondary structure of the protein (Cavanagh et al. 2007; Wishart & Case 2001). By comparing the $\Delta \delta$s of the different main chain atoms of the same amino acid residue, it is possible to get a robust determination of the secondary structure for that residue.

In addition to observing the four transmembrane helices (S1-S4), by examining the $\Delta \delta$s of the hHvΔNΔC construct in LPPG we experimentally confirmed the existence of a short N-terminal helix (S0) that precedes the first transmembrane helix S1 (approximately 10 amino acid residue long, R89-S98; Fig. 5.8). Although an analogous S0 helix has been reported in the NMR structure of the isolated VSD of KvAP (Butterwick & Mackinnon 2010), this was the first observation of this structural feature in Hv channels.

Based on the $\Delta \delta$ observations, we are able to estimate the amino acid residue span of each of the transmembrane helices. According to these data, S1 spans residues V103-D123, S2 starts at residue N133 and continues at least until residue L163, S3 extends at least from residue D174 to L188 and S4 spans residues F195-S219 (Fig. 5.8). The ambiguity in the start position of helix S3 and stop position of helices S2 and S3 derives from the lack of assignments for main chain atoms from amino acid residues within the S2-S3 and S3-S4 loops. Nonetheless, it is clear from these data that the secondary structure of hHvΔNΔC in LPPG matches what would be expected for a VSD.
Fig. 5.8 Secondary structure of hH\textsubscript{V}\Delta\textsubscript{N}\Delta\textsubscript{C}

The VSD helices are identified by the pattern of secondary chemical shifts for $^{13}\text{C}^{'}, ^{13}\text{C}^\alpha$, \[\Delta\delta(^{13}\text{C}^X);\] corrected for $^2\text{H}$ isotope shifts (Venters \textit{et al.} 1996) and graphed from $-5$ ppm to $+5$ ppm and $^1\text{H}^\alpha$ and $^1\text{H}^N$ \[\Delta\delta(^1\text{H}^X);\] graphed from $-1$ ppm to $+1$ ppm (Wishart & Case 2001). The helices identified are labeled at the top of the plot and the red arrows to the right of each plot indicate the direction of $\Delta\delta$ that correspond to helical structure.
5.2.5 Through-space measurements indicate a poorly defined tertiary structure

In order to solve the three dimensional structure of the channel, we needed to measure long-range through-space interactions between the different transmembrane helices. The $^{13}$C-edited and $^{15}$N-edited NOESY experiments gave us some distance restraints; however, due to crowding in the aromatic region of the spectra, we were unable to assign many of the interactions. Therefore, we performed a series of specific labeling experiments for paramagnetic relaxation enhancement (PRE), to better constrain the overall fold and thereby help with the assignments of the observed NOESY peaks.

The electron spin of a stable nitroxide free radical enhances the relaxation of nearby nuclei in a magnetic field (Hilty et al. 2004). By introducing a cysteine mutation at a specific site in a cysteine-free background (i.e. C107S) and labeling the cysteine with a paramagnetic nitroxide-free-radical-containing small molecule, it is possible to measure a decrease in the peak intensity of nearby nuclei. This decrease in peak intensity is proportional to the distance between the nucleus and the paramagnetic probe. Through comparison of the spectrum of the paramagnetic-labeled protein to a control spectrum in which the cysteine is modified with a diamagnetic version of the small molecule, it is possible to accurately calculate long-range distances (15-20 Å) between the labeled position and other parts of the protein. These long-range distances can then be added as constraints into a structure calculation.

In order to generate long-range distance constraints for the hHvΔNΔC construct, we labeled the channel at five different positions: R100C at the N-terminus of S1; I127C and Y134C in the S1-S2 loop; L173C in the S2-S3 loop and F190C in the S3-S4 loop (Fig. 5.9). The PRE data presented in Fig. 5.9A indicate that the channel was efficiently
labeled at each position. However, significant long-range signal suppression was not apparent. In contrast, when an equivalent position in the S3-S4 loop was labeled on the homologous KvAP isolated VSD, strong signal suppression was seen for nuclei on the S1-S2 loop, which is what would be expected for a four-helix bundle (Fig. 5.9C). The lack of strong PRE signal for the hHvΔNΔC construct indicates the tertiary structure of the channel in LPPG may be poorly defined. In fact, the only suppression observed was between positions on the S1-S2 loop (I127 and Y134) and the C-terminus of S4 (Fig. 5.9A). In the native fold of a VSD, these two regions of the protein should be on opposite sides of the membrane and therefore we would not expect to see any PRE signal between them. The fact that we did see this suppression at both S1-S2 positions, strongly suggests that the hHvΔNΔC construct in LPPG was in a non-native conformation.

Our inability to measure strong distance constraints in the hHvΔNΔC construct and our observation of likely non-native interactions between the S1-S2 loop and the C-terminus of S4 led us to conclude that structure determination of the channel by NMR under these conditions was infeasible. This approach was therefore abandoned.
Fig. 5.9 PRE experiments on hHΔNΔC

(A) PRE data for the different specifically labeled positions along the hHΔNΔC construct. Data are presented as the ratio of peak intensity for the spin-labeled sample (I_{sl}) over the peak intensity for the diamagnetic-labeled sample (I_{dia}) versus amino acid residue number. In each case the red arrow indicates the site of the label. The solid red curves indicate the expected intensity drop near the label site for an extended random coil structure, whereas the dashed red line indicates the expected intensity drop near the label site for a more compact helical structure. (B) Schematic of hHΔNΔC topology indicating the positions of the labeling sites. (C) Data for the K_{v}AP isolated VSD with labels at equivalent positions on S1-S2 loop (Y46 in K_{v}AP equivalent to I127 in H_{v}) and S3-S4 loop (F116 in K_{v}AP equivalent to F190 in H_{v}) demonstrating the expected signal suppression between the two “extracellular” loops that would be expected for a four-helix bundle structure. K_{v}AP data provided by Dr Joel Butterwick from Butterwick & Mackinnon 2010.
5.3 The Structural Necessity of the Membrane

The biochemical characterization of the different hH\textsubscript{\text{V}}\textsubscript{1} and chimeric constructs presented in this thesis clearly demonstrates the importance of the lipid bilayer membrane to the maintenance of the native structure of H\textsubscript{\text{V}} channels. Before pursuing the structure of each of the constructs, we had ensured that they were functional by electrophysiology and again after purification by reconstitution and characterization in the flux assay. However, in all these functional and cross-linking studies, the channels were embedded within a lipid bilayer (either that of a cell membrane or of reconstituted lipid vesicles). In contrast, all structural work was performed on detergent-solubilized channels, i.e. in the absence of lipid membranes. As described throughout the thesis, in every case, these previously functional channels adopted a non-native conformation in detergent.

It is clear, then, that although the channel protein is stable in some detergents the channel structure is unstable when removed from the membrane and that future directions for structural work on H\textsubscript{\text{V}} channels must focus on membrane-like conditions. Additional crystallography attempts, including future work using the phage-display Fabs discussed in Chapter 4, must focus on detergent-lipid mixtures, bicelle and lipidic cubic phase conditions. Any future work by solution state NMR should only be performed on H\textsubscript{\text{V}} channels incorporated into lipid nano-disks. It may also be possible to try solid-state NMR on pellets of membranes containing reconstituted H\textsubscript{\text{V}} channels.
MATERIALS AND METHODS

Preparation of cDNA and mutations for HEK cell transfections

Human H\(_\nu\)1 cDNA (GI: 34783431, a gift from David Clapham, Harvard University) was subcloned into pcDNA4 vector (Invitrogen). Mutations were generated using QuikChange kit (Stratagene). Three additional amino acids (ARG) were introduced into the C-terminus as a byproduct of cloning into the expression vector. These amino acids were later removed for all constructs used for electrophysiological recordings.

Electrophysiological recordings from HEK and CHO cells

Full-length hH\(_\nu\)1 (Fig. 1.6, 4.15 and 5.3), the HAP5 chimera (Fig. 4.15) and the truncated constructs (Fig. 5.3) used for crystallization and NMR were cloned into a pcDNA4 vector (Invitrogen) for mammalian cell expression. All constructs used for recordings shown in Fig. 1.6 and 5.3 contained N-terminal GFP fusions. The hH\(_\nu\)1 and HAP5 constructs used for recordings in Fig. 4.15 had no GFP fusion but were co-transfected with a GFP containing vector in order to facilitate detection of transfected cells. HEK tsA201 cells (ATCC) were maintained in DMEM (Gibco) containing 10% FBS. Cells were transfected using FuGene (Promega) following the manufacturers protocol then, after ~6 hours, were split onto poly-D-lysine-coated glass coverslips (BD BioCoat) and allowed to recover for
~12-24 hrs. After 12-48 hrs, coverslips were transferred to the recording chamber. Immediately before recording, medium was replaced by bath solution. All recordings were performed at room temperature. Recordings were obtained with an Axopatch 200B amplifier (Molecular Devices) using standard whole-cell patch-clamp techniques. Recordings were filtered at 1 kHz with sampling at 10 kHz. Pipettes of 2-5 MΩ resistance were pulled from borosilicate glass and fire polished. Currents were recorded according to the voltage step protocols represented schematically in each figure.

Pipette solution for recordings shown in Fig. 1.6 and 5.3 was: 100 mM Tetramethylammonium chloride (TMACl), 2 mM MgCl₂, 100 mM MES (pH 6.5) and 5 mM Glucose (353 mOsm). Bath solutions for recordings shown in Fig. 1.x and 5.x were: 100 mM TMACl, 2 mM MgCl₂, 100 mM MES (pH 6.0) and 25 mM Glucose (352 mOsm); 100 mM TMACl, 2 mM MgCl₂, 100 mM MES (pH 6.5) and 5 mM Glucose (353 mOsm); 100 mM TMACl, 2 mM MgCl₂, 100 mM HEPES (pH 7.0) and 50 mM Glucose (356 mOsm). All solutions had pH adjusted using N-methyl-D-glucamine (NMDG).

Pipette solutions for recordings in Fig. 4.15 was: 64 mM NMDG, 3 mM MgCl₂, 1 mM EGTA and 150 mM MES (pH 6.5). The bath solution was: 75 mM NMDG, 1 mM MgCl₂, 1 mM CaCl₂ and 100 mM HEPES (pH 7.5). These solutions had their pH adjusted with methanesulfonic acid and osmotic concentration matched by addition of glucose.

The recordings shown in Fig. 1.2 were provided by Josefina del Mármol and were produced by the whole-cell patch clamp technique described above with slight modifications. The Rattus norvegicus Kᵥ1.1 (GI: 24520) subcloned into a pcDNA3.1 vector containing an IRES mCherry was used to transiently transfect CHO cells (ATCC)
by means of lipofectamine 2000 (Life Technologies) according to the manufacturers protocol. Pipette solution was: 150 mM KCl, 10 mM HEPES (pH 7.4), 2 mM MgCl₂ and 5 mM EDTA, pH’ed with KOH. Bath solution was: 135 mM NaCl, 15 mM KCl, 10 mM HEPES (pH 7.4), 2 mM MgCl₂, and 1 mM CaCl₂, pH’ed with NaOH.

Membrane preparation

cDNAs encoding hHv1 were transfected into tsA201 (HEK293 derivatives) cells using Lipofectamine 2000 (Invitrogen) for 36-48 h. Membranes were prepared as described with modifications (Asano et al. 1996). Briefly, cells were washed with phosphate buffered saline with 1 mM EDTA and lysed with a tissue grinder (wheaton) in the presence of protease inhibitors (1 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 1 mg/ml pepstatin). Lysed cells were briefly sonicated for 10 s in a bath sonicator and then centrifuged at 900 g for 15 min. Supernatants were collected and diluted 4-fold with ice-cold buffer (20 mM HEPES, pH 7.5, 5 mM KCl, 150 mM NaCl, 5 % glycerol, and 1 mM EDTA) and ultra-centrifuged for 90 – 120 min at 130,000 g. After ultra-centrifugation, supernatants were discarded and pellets were resuspended in the same buffer and then homogenized with a tissue grinder (wheaton). Samples were maintained at 4°C at all times.

Cross-linking and Western blotting

For non-specific cross-linking experiments, the amino-group reactive bifunctional cross-linker DSS (Pierce) was used. For each reaction, 20-fold concentrated DSS stocks,
dissolved in dimethylsulfoxide (DMSO), were added to prepared membranes and incubated at room temperature for 20 min; reactions were quenched by the addition of Tris-HCl (pH 8.5) to a final concentration of 100 mM. For copper mediated cross-linking, 6 mM CuSO$_4$ and 1.8 mM o-phenanthroline in water were added to prepared membranes to a final concentration of 300 µM and 900 µM, respectively and incubated at room temperature for 20 min; reactions were quenched by addition of 20 mM N-ethylmaleimide (NEM) and 50 mM EDTA. For cysteine-directed cross-linking, 1 mM stock of 1,3-propanediyl bismethanethiosulfonate (M3M, Toronto Research Chemicals Inc) in DMSO was added to the prepared membranes to a final concentration of 50 µM and incubated on ice for 1 hr; reactions were quenched with 20 mM NEM. Air-oxidized samples, used immediately after membrane preparation, were treated with 10-20 mM NEM to prevent cross-linking during electrophoresis. All the samples were mixed with equal volume of loading buffers containing 4 % (w/v) SDS and 10 % (v/v) β-mercaptoethanol (only for reducing condition), then subjected to SDS-PAGE on 12 % gels, transferred onto polyvinylidene difluoride (PVDF) membrane, and probed with a mixture of two monoclonal antibodies 25H11 and 9C1. Monoclonal antibodies were raised in mice injected with purified hHv1 protein by standard protocols (Harlow & Lane 1988).

Expression of hHv1 in Pichia Pastoris

The gene for the full-length human H$_v$ channels (GenBank accession no: 91992153) with a C-terminal 1D4 tag (ARAAGGTTENTSQVAPA) was ligated into the PICZ-c vector (Invitrogen Life Technologies). This vector was transformed into a His$^+$ strain of
SMD1163 *Pichia pastoris* and selected as described (Long S. B., *et al.*, 2005). Transformed cells were grown in 1 L cultures of BMG media (Yeast Nitrorgen Base, 100 mM sodium phosphate pH 6.3 and 1% glycerol) at 30°C until an optical density of ~20 was reached. BMG media was exchanged for BMM media (BMG with 0.75-1% MeOH instead of glycerol) and grown at 24-27°C for 12-24 hours. Cell pellets were frozen in liquid nitrogen and stored at -80°C until needed. This same expression protocol was used for the chimeric-1D4 and N-terminal-GFP full-length and truncated chimeric and wild type channels.

For expression of $^{15}$N labeled protein all ammonium sulfate in the media was replaced with $^{15}$N ammonium sulfate. For $^{13}$C labeled samples glycerol in the BMG media and methanol in the BMM media were replaced by $^{13}$C-glycerol and $^{13}$C-methanol respectively. In order to promote maximal expression the cells grown in 70-90% D$_2$O BMG were only allowed to reach an optical density (measured at 600 nm) of ~5. They were then spun down and resuspended into a half volume of 70-90% D$_2$O BMM (concentrating the cells twofold) and incubated at 27°C for 16-24 h before harvesting.

Expression of GFP-hH$_v$ΔNΔC in *E. coli* for specific amino acid labeling

To produce amino-acid-specific and methyl-specific labeling patterns samples, we grew XL-1 Blue cells (Stratagene), which were transformed with an expression vector, in LB broth at 37°C until the optical density (measured at 600 nm) had reached ~ 0.8 The cells were then centrifuged for 10 min at 3000 x g and resuspended in a half volume of M9 minimal medium (concentrating the cells twofold) supplemented with and 10 mg/L thiamine and the isotopically enriched amino acid (sodium salt) at 50–100 mg/L, and all
nonlabeled amino acids were included at 100–200 mg/L. Similarly, to specifically label the Ile$^{\delta_1}$ and/or Leu$^{\delta_2}$/Val$^{\gamma_1}$ groups (denoted $^{13}$C-methyl), we added 50 mg/L sodium 2-keto-4-13C-butyrate (for Ile) and 100 mg/L sodium 2-keto-3-methyl-$d_7$-4-13C-butyrate (for Leu/Val) in lieu of their respective amino acids (Tugarinov et al. 2006). It should be noted that for Leu$^{\delta_2}$ and Val$^{\gamma_1}$ methyl groups labeled in this manner, one group within the pair is $^{13}$CH$_3$, while the other is $^{12}$CD$_3$. After 1 h, protein expression was induced by the addition of 0.5 mM isopropyl-$\beta$-D-thiogalactopyranoside, and the cells were harvested 12–16 h later.

1D4-purification

Frozen pellets were lysed with a mixer mill (Retsch, Inc. Model MM301) and resuspended in buffer (500 mM NaCl, 50 mM TRIS-HCl, pH 8.5, 2 mM $\beta$-mercaptoethanol, 0.1 mg/ml deoxyribonuclease I, 1 $\mu$g/ml pepstatin, 1 $\mu$g/ml leupeptin, 1 $\mu$g/ml aprotinin, 1.0 mM phenylmethysulfonyl fluoride and 2.0 mM Ethylenediaminetetraacetic acid (EDTA). The pH was adjusted to 8.5 with NaOH, and 0.15 g DDM (n-dodecyl-$\beta$-D-maltopyranoside, Anatrace) per g of cells was added prior to a 2-3 hour extraction at room temperature followed by centrifugation at 31000 x g for 25 min. Supernatant was added to 1D4 antibody-linked sepharose affinity resin previously equilibrated with buffer A (500 mM NaCl, 50 mM TRIS-HCl (pH 7.5), 1 mM EDTA and 1 mM DDM) and rotated at room temperature for 2 hours. The resin was collected on a column, washed with buffer A (4 x 5 column volumes) and eluted with buffer A containing 0.4 mg/ml 1D4 peptide. Protein was loaded on a Superdex-200 gel filtration column in 20 mM TRIS-HCl (pH 7.5), 150 mM KCl, 50 mM NaCl and 4 mM
DM (n-decyl-β-D-maltopyranoside, Anatrace, anagrade; Buffer B). The fractions corresponding to Hv channels were pooled.

**Reconstitution of hHv1 channels**

A mixture of 6:6:3:3:1 of POPC:POPE:POPS:SM:PI (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine, 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphoethanolamine, 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phospho-L-Serine, Sphingomyelin, and L-a-Phosphatidylinositol, obtained from Avanti) was prepared based on the composition of human neutrophil plasma membrane (Tamura *et al.* 1988). A mixture of 3:1 POPE:POPG can also be used. The lipid mixture was dried under an Argon stream and then resuspended to 10 mg/ml in dialysis buffer (20 mM HEPES (pH 7.0), 150 mM KCl, 10% glycerol, 0.2 mM EGTA and 2 mM 2-mercaptoethanol). The lipid mixture was then sonicated in a bath sonicator three times for 2 minutes. Decylmaltoside (DM) was added to the lipid mixture to 10 mM and rotated at room temperature for 1hr. Protein was added to the lipid mixture in a 1:100 (wt:wt) protein to lipid ratio and an additional 10 mM DM was added. As a control empty vesicles were made in which only dialysis buffer was added to the lipids. The protein-lipid mixture was rotated at room temperature for ~3 hours then placed into dialysis membrane (molecular weight cut off of 50 KDa) and dialyzed in 4 L of dialysis buffer for 5 days at RT exchanging buffer daily. Vesicles were then harvested and flash frozen in liquid nitrogen and stored at -80°C until needed.
Fluorescence based flux assay

The fluorescence data for vesicles containing H_\text{v} channels was obtained using a published procedure with the following modification (Zhang & Forgac 1994). Vesicles were thawed in room temperature water and then sonicated once in a bath sonicator for 5 seconds and then diluted 20 fold into flux buffer (20 mM HEPES (pH 7.0), 150 mM NaCl, 7.5 mM KCl, 10% glycerol, 0.2 mM EGTA, 0.5 mg/ml BSA, 2 mM 2-mercaptoethanol and 2 mM ACMA) in a quartz cuvette. Data were collected on a Spex Fluorolog 3-11 spectrofluorometer in time acquisition mode at 30-second intervals with excitation at 410 nm, emission 490 nm, with bandwidth 5 nm and an integration time 2 s. A baseline was collected for 150 s before the addition of 20 nM valinomycin. After the fluorescence stabilized carbonyl cyanide m-chloro phenyl hydrazone (CCCP) was added to 2 mM rendering all vesicles H^+ permeable and a minimum baseline was collected for 150 seconds. Data are scaled by \((F_i - F_{\text{min}})/(F_{\text{max}} - F_{\text{min}})\), where \(F_{\text{max}}\) is the average value of the starting baseline and \(F_{\text{min}}\) is the average value of the minimum baseline. \(F_{\text{max}} - F_{\text{min}}\) (the total reduction in fluorescence after CCCP addition) was ~ 25 % for all vesicles.

Sucrose Gradient Flotation Assay

Lipid vesicles containing hH_{\text{v}}1, with protein to lipid ratio 1: 100 (wt:wt), were layered on a sucrose gradient (From top to bottom, 140 µl sample plus 60 µl dialysis buffer, 600 µl 7% sucrose, and 1 ml 27% sucrose in dialysis buffer). The gradients were then centrifuged at 135,000 x g in a Sorvall RP55-S swinging bucket rotor for 2 hours and then fractionated into 8 x ~225 µl fractions. A 15 ml sample of each fraction was then
mixed with 15 ml 2x running buffer and run on a 12% gel (SDS-PAGE) and stained with Coomassie blue.

Mass Spectrometry

Mass spectrometry was performed by the ultra-thin layer method according to published protocols (Cadene & Chait 2000). In short protein samples were concentrated to 1 mg/mL then diluted with FWI (3 parts formic acid, 1 part water, 2 parts isopropanol) into 10X final concentration aliquots which were further diluted (1 to 10) with saturated acyano-4-hydroxycinnamic acid (4-HCCA) in FWI to final ratios of 1:10, 1:20, 1:50, 1:100 and/or 1:200. 0.5 µL of each sample was spotted onto a stainless steal MALDI sample plate coated with an ultrathinlayer of 4-HCCA. After a visible precipitate had formed on the bottom of the spots the excess liquid was aspirated away and the spots were washed with 2 µL of 0.1% trifluoro acetic acid (TFA) solution. The samples were then taken for analysis. All spectra were aquired using MALDI time-of-flight mass spectrometer Voyager-DE STR (PE Biosystem, Foster City, CA) operating in linear, delayed extraction mode. This instrument is equipped with a nitrogen laser delivering pulses of ultraviolet light (wavelength 337 nm) at 3 Hz to the matrix spot. Spectra from 200-500 individual laser shots were averaged (using 2-ns data channel width) with software provided by the manufacturer. The spectra were smoothed, calibrated, and analyzed using the program M-over-Z (http://www.proteometrics.com and http://prowl.rockefeller.edu). For more details see:

http://prowl.rockefeller.edu/protocols/ultra-thin-layer.html
decaHis-GFP- construct purification

Frozen pellets were lysed with a mixer mill (Retsch, Inc. Model MM301) and resuspended in buffer (500 mM NaCl, 50 mM TRIS-HCl, pH 8.5, 2 mM β-mercaptoethanol, 0.1 mg/ml deoxyribonuclease I, 0.1 mg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1.0 mM phenylmethysulfonyl fluoride). The pH was adjusted to 8.5 with NaOH, and DM (n-dodecyl-β-D-maltopyranoside, Anatrace) was added to a final concentration of 80 mM prior to a 1.5 hour extraction at room temperature followed by centrifugation at 31000 x g for 30 min. Supernatant was added to Co²⁺ Talon (CloneTech) IMAC resin equilibrated with buffer A (500 mM NaCl, 50 mM TRIS-HCl, (pH 7.5) 5 mM imidazole and 4 mM DM) and rotated at room temperature for 2 hours. The resin was collected on a column, washed with buffer A (4 x 5 column volumes), further washed with buffer A containing 25 mM imidazole and eluted with buffer A containing 250 mM imidazole.

For the decaHis-GFP-HAP5ΔNΔC construct 2 mM DTT and PreScission protease were added the elution and the sample was incubated at 4°C overnight followed by a second round of Talon resin purification in buffer A from which the flow through was collected and concentrated. This sample was loaded on a Superdex-200 gel filtration column in 20 mM TRIS-HCl (pH 7.5), 200 mM NaCl and 4 mM DM (Buffer B). The fractions corresponding to HAPΔNΔC channels were pooled and concentrated.

For the decaHis-GFP-hHvΔNΔC construct 2 mM DTT and PreScission protease was added the elution and the sample was incubated on ice for 1 h before being added to 15 kDa MWCO dialysis membrane (Spectrum Labs) and dialyzed overnight at 4°C into low salt buffer (depending on the volume of the decaHis-GFP-hHvΔNΔC sample, 400
mL of 12.5-30 mM NaCl, 10 mM BisTris (pH 7.0), 2 mM DTT and 4 mM DM) for a final concentration of 50 mM NaCl. The sample was then applied to a Q-sepharose column equilibrated in 50 mM NaCl, 10 mM BisTris (pH 7.0), 2 mM DTT and 4 mM DM buffer. The flow through from the Q-column was collected, concentrated and loaded on a Superdex-200 gel filtration column in 10 mM HEPES, pH 7.0, 50 mM NaCl and 2 mM LPPG or DHPC. The fractions corresponding to HAPΔNΔC channels were pooled and concentrated.

33H1 and 6E1 Fab purification

Antibodies were prepared as described in Brohawn et al. with slight modifications (Brohawn et al. 2013). Briefly, media supernatant from hybridomas was dialyzed against two changes of 4 L of 10 mM Tris (pH 8.0), 10 mM NaCl in 8-kDa-MWCO dialysis tubing (Spectrum Labs) overnight. Dialyzed samples were spun at 6,000 × g, and the supernatant was applied to a 5-mL Q-Sepharose column (GE Healthcare) equilibrated in 10 mM Tris (pH 8.0), 10 mM NaCl. Antibodies were eluted during a gradient to 10 mM Tris (pH 8.0), 1.0 M NaCl. Eluted antibodies were diluted to 3 mg/mL in PBS. Fab fragments were generated by reaction with papain (1:100 wt:wt) in PBS with 10 mM β-mercaptoethanol, 10 mM L-cysteine HCl, and 10 mM EDTA (pH 7.0) at 37°C for 4 h. Cleaved antibodies were dialyzed against two changes of 4 L of 10 mM Tris (pH 8.0), 10 mM NaCl in 8-kDa-MMCO dialysis tubing overnight. Dialyzed samples were spun at 6,000 × g, and the supernatant was applied to a 5-mL Q-Sepharose column equilibrated in 10 mM Tris (pH 8.0), 10 mM NaCl. Fab fragments were collected in the flow-through.
Crystallization and structure determination of HAP5ΔNΔC/33H1

Pure HAP5ΔNΔC was mixed with pure 33H1 Fab and concentrated (10kDa MWCO, Millipore) to ~15 mg/mL total protein for crystallization. 0.4 µL protein was added to 0.4 µL reservoir (30% PEG400) in hanging drops. The largest crystals appeared within 1 week and grew to full size in 2-3 weeks at 20° C. Crystals were harvested and frozen in liquid nitrogen.

Data were collected at NSLS beamline X29 and processed with HKL2000 (Minor et al. 2006). Data were anisotropic and were elliptically truncated and scaled (Strong et al. 2006) to 3.9 x 5.1 x 3.8 Å. Molecular replacement solution was found using the program Phaser (McCoy et al. 2007) and the 33H1 Fv and Fc as search models. Crystallographic programs from the Phenix and CCP4 suites were used throughout structure determination (Adams et al. 2010; Winn et al. 2011). Structure figures were generated with Pymol (Schrödinger LLC).

Phage Display Selections

Were performed as described in sections 4.3.1 and 4.3.2 and Sidhu et al. 2000. In brief, biotinylation of purified hHvΔNΔC was performed at 1 mg/mL protein in 200 mM NaCl, 20 mM HEPES (pH 7.0), 5 mM DHPC (Buffer PD). For cysteine specific biotinylation final concentration of 2 mM Biotinylcaproylaminocaproyl-aminoethyl Methanethiosulfonate (BCAC; Toronto Research Chemicals, Inc.) was added to the protein and incubated for 3-4 h at room temperature in the dark, excess BCAC was removed by SEC. For amine specific biotinylation N-hydroxysuccinimidyl d-biotin-15-
amido-4,7,10,13-tetraoxapentadecylate (NHS-PEO4-Biotin; Thermo Scientific) was added in a molar ratio of 1:3 hHΔNΔC: NHS-PEO4-Biotin and incubated at 4°C, samples were used directly without removal of excess reagent.

Detergent based selections: Pre-absorption (negative selection) and selection wells on the 96-well plate (for each selection [positive and negative] the number of wells was: round 1, 8 wells; round 2, 6 wells; round 3, 4 wells; round 4, 2 wells; round 5, 2 wells) were coated overnight at 4°C with 100 µL of 5-25 µg/mL streptavidin or neutravidin in 200 mM NaCl, 20 mM HEPES (pH 7.0) then blocked with 200 µL of 0.5% BSA for 1 h at room temperature. For selections 100 µL of biotinylated hHΔNΔC was added to the positive selection wells at 10 µg/mL in buffer PD and incubated at 4°C for 1h at the same time 100 µL phage library at 10^{12}-10^{13} cfu/mL buffer PD was added to the negative selection wells and incubated at 4°C for 1h. hHΔNΔC solution was removed from positive selection wells and unbound phage were transferred from negative to positive selection wells and incubated at 4°C for 2h. Phage solution was removed from the positive selection wells and the wells were washed with 6X for round 1, 8X for round 2, 10X for round 3 and 15X for rounds 4 and 5 with buffer PD. Bound phage were eluted by adding 100 µL/well of 100 mM HCl and incubating for 5 min at room temperature with shaking. The HCl solution was then added to an eppendorf tube containing 25 µL 1 M Tris-HCl (pH 8) per well (8 wells = 200 µL Tris + 800 µL phage) then 500 µL of this phage solution was added to 7.5 mL of actively growing OPTIMAX cells (OD = 0.5-0.8) in 2YT. Infected OPTIMAX cells were incubated for 30 min at 37°C with shaking then M13K07 helper phage were added to get 10^{10} cfu/ml final and then further incubation for 45 min at 37°C with shaking. The culture was transferred to 35 mL final volume
2YT/carb100/kan25 medium and incubated overnight at 37°C with shaking. Overnight phage cultures were precipitated: spun cultures in 50 ml Falcon tubes 8000 rpm, 15 minutes, added 8.75 ml (1/5 final volume) PEG/NaCl (20% PEG 8000 w/v, 2.5 M NaCl) to supernatant, incubated on ice for 20 minutes, spun 20 min at 24000 g, re-suspended pellets in 1.2 mL volume of buffer PD and transfer to eppendorf tubes, spun again (5 min, 13000 rpm) to remove bacterial debris then transferred supernatant to new tubes (this is input phage for next round of selections). After each round input and output phage were titered: 10 µL of 10-fold serial dilutions of precipitated phage (10 µL phage+ 90 µL 2YT), were added to 90 µL OPTIMAX (OD600 0.5-0.8), incubated for 30 min at 37°C, and then 5 µL of each dilution were spotted plates (carb, kan, tet), final dilutions of 10^2 to 10^7 and the 10^6 to 10^11 were used for output and input phage respectively. Plates were incubated overnight at 37°C and colonies were counted the next day.

**Vesicle based selections:** Performed as for the detergent based selections with the following modifications. Antigen buffer (AB) used was 150 mM KCl, 20 mM HEPES (pH7.0), 5% glycerol. DPPE-biotin-containing empty vesicles were added to the negative selection wells prior to the addition of input phage (see section 4.3.2). The unbound phage from the negative selections were mixed with DPPE-biotin-containing hH₁ΔNΔC vesicles for 1 h at 4°C with shaking prior to quick capture of vesicles and bound phage on streptavidin or neutravidin coated wells (15 min at 4°C) followed by washing and elution.

**Isolation of positive binders:** The output phage from the final round of selections were mixed with 7.5 mL of actively growing OPTIMAX cells (OD = 0.5-0.8) in 2YT for 30 min at 37°C then diluted and plated onto LB plates/carb100 in order to grow single colonies and incubated overnight at 37°C. Single colonies were picked (96-192 per
library), used to inoculated individual 2 mL cultures of 2YT/carb100 and incubated at 37°C with shaking until OD600 0.5-0.8, then M13K07 helper phage were added and the cultures were incubated overnight at 37°C with shaking. These cultures were spun down to pellet bacterial cell debris and the supernatant was used in ELISAs (like the one shown in Table 4.3). In brief, ELISA plates were coated with streptavidin and blocked with BSA in the same way as the selection plates, four wells were used per the isolated phage clone: BSA alone, empty vesicles and two wells containing hH\(_{\alpha}\)ΔNΔC vesicles. Supernatant from isolated phage cultures were diluted 2-fold with 2 X AB and incubated with antigen (either in detergent or in vesicles) for 1 hr at 4°C with shaking prior to quick capture of biotin on the plates (15 min 4°C with shaking), followed by washes and incubation with primary antibody (either αFLAG or αM13 see Table 4.3). ELISAs were then developed by standard protocols. Isolated phage clones that were positive for binding were sequenced then subcloned into an expression vector for Fab expression and purification.

**Phage display Fab expression and purification**

Single colonies from *E. coli* BL21 DE3 transformed with the phagemid DNA containing the Fab sequence was grown at 37°C in 25 mL 2YT/amp medium for ~3 hours then used to inoculate 1 L of 2YT/amp media and grown at 37°C until an optical density (measured at 600 nm) of 0.6 was reached. Expression was induced by the addition of 1 mM IPTG and the cells were left overnight at 37°C. Cells were pelleted by centrifugation for 10 minutes at 3,000 x g then frozen in liquid nitrogen and stored at -80°C.

Cell pellets were resuspended in lysis buffer (100 mM NaCl, 20 mM Tris (pH 8.0), 0.1 mg/ml deoxyribonuclease I, 0.1 mg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml
aprotinin, 1.0 mM phenylmethylsulfonyl fluoride) and lysed by sonication. Cell debris
was removed by centrifugation for 40 min at 12,000 rpm. Supernatants were mixed with
2-3 mL of Talon Co²⁺ resin (CloneTech) equilibrated in wash buffer (100 mM NaCl, 20
mM Tris (pH 8.0) and 5 mM imidazole) and tumbled for 1-2 h at room temperature. The
resin was collected on a column, washed with wash buffer (4 x 5 column volumes), and
eluted with wash buffer containing 250 mM imidazole. Sample was then dialyzed into
low salt buffer A (10 mM NaCl, 10 mM Tris (pH 8.0)) overnight and then run over a Q-
sepharose column. The Q-sepharose flow through was collected and concentrated for use
in binding experiments.

NMR data collection and analysis

NMR experiments were performed at the New York Structural Biology Center using
Bruker Avance or Avance II instruments operating at static magnetic field strengths of
14.1, 18.8 and 21.1 T, equipped with z-shielded gradient triple-resonance TCI or TXI
cryogenic probes. The sample temperature was maintained at 25°C during the initial
screening of detergent and buffer conditions and for all other experiments. NMR spectra
were processed using the NMRPipe software package (Delaglio et al. 1995) and analyzed
using the program SPARKY (Goddard & Kneller).

Chemical Shift Assignments

Resonance assignments for backbone \(^1\text{H}^N\), \(^{15}\text{N}\), \(^{13}\text{C}'\), and \(^{13}\text{C}^\alpha\), and \(^{13}\text{C}^\beta\) nuclei were
identified using three-dimensional (3D) TROSY HNCA (at 21.1 T), HNCO, HN
(CO)CA, and HNCACB (at 18.8 T) experiments (Cavanagh et al. 2007; Neri et al. 1989) performed on 0.15-0.3 mM ²H, ¹³C, ¹⁵N samples. Also, two-dimensional (2D) TROSY HSQC and 3D ¹⁵N-edited NOESY (mixing times $\tau_{\text{mix}} = 80$) experiments (at 21.1 T) were recorded on a 0.3 mM ²H, ¹⁵N sample. In addition to uniformly labeled samples, 2D HSQC, HNCA, and HNCO experiments (at 18.8 T) were recorded on 0.3 mM samples with varied amino-acid-specific labeling patterns designed to eliminate ambiguities as discussed in section 5.2.3.

Side-chain resonance assignments were based on 3D HC(C)H-COSY ¹³C-edited (aromatic and aliphatic) and ¹⁵N-edited ($\tau_{\text{mix}} = 80$ ms) NOESY experiments (at 21.1 T) recorded on 0.5 mM ¹³C, ¹⁵N samples in 99.9% (vol/vol) D₂O and on a 3D ¹⁵N-edited ¹H–¹H NOESY ($\tau_{\text{mix}} = 80$ ms) experiment (at 21.1 T) recorded using a 0.4 mM ¹⁵N sample. To improve resolution within the Val and Leu methyl regions, we recorded a 3D ¹³C-edited NOESY ($\tau_{\text{mix}} = 100$ ms) experiment on a ¹³Cmethyl-LV sample.
APPENDIX I

This Appendix contains the MATLAB code for my model of H\(^+\) flux into vesicles reconstituted with hH\(_V\)1 channels based on the equivalent circuit model found in Moffat et al. 2008 (see Chapter 3). Green text that follows a ‘%’ indicate comments which I have used to annotate the code. If you are looking at a PDF file it should be possible to copy and paste the code along with the other functions below into separate text files and save them as simple_flux_V_Na_Cl.m, pt_ffluxbias.m, total_proportional_flux_V.m, channel_dist_theta.m and unscaled_plot.m then use MATLAB to execute the code by typing the command simple_flux_V_Na_Cl(30) in the command window. Currently the conductances for Na\(^+\) and Cl\(^-\) are set to zero but this can be adjusted by altering the highlighted variables GNa and GCl.

```matlab
function simple_flux_V_Na_Cl(m)
%calculates the basis set of fluxes for a series of 1 to m
%channels per vesicle
%the basis set is to be use in the programs pt_ffluxbias or
%total_proportional_flux_V
%through their global assignmen

global time1 %assigns the time vector as a global variable
global sflux %assigns the sflux matrix as a global variable
global fflux %assigns the fflux matrix as a global variable

Ki1 = 0.150; %initial internal concentration of K+ in M
Ko1 = 0.015; %initial outer concentration of K+ in M
Hi1 = 1e-7; %initial internal concentration of H+ in M
Ho1 = 1e-7; %initial outer concentration of H+ in M
Nai1 = 1e-4; %initial internal concentration of Na+ in M
Naol = 0.150; %initial outer concentration of Na+ in M
Cl1l = 0.150; %initial internal concentration of Cl- in M
Cloc1 = 0.165; %initial outer concentration of Cl- in M
Vo = 0.0008; %bath volume
Vi = 2.3562e-18; %volume inside average vesicles (r = 100 nm)
F  = 96485; %Faraday constant 9.6485e4 C/mol
R  = 8.3145; %gas constant 8.3145 V*C/mol*K
T  = 298; %absolute temp 298 K
BKd = 0.000000028183829; %dissociation constant for HEPES buffer pKa 7.55
Bi = 0.02; %internal concentration of HEPES buffer
Bo = 0.02; %outer concentration of HEPES buffer
GK = 1e-6; %k+ conductance through the membrane
```
Vmid = 0.04; %V1/2 of voltage sensor Boltzmann
z = 3; %assigns valence for hHvl voltage sensor Boltzmann
n = 2000000; %number of iterations of loop
dt = 0.001; %time step per iteration in seconds
%duration of time = n*dt

%builds fflux and sflux matrices
fflux = zeros(m,n+1);
sflux = zeros(m,n+1);
for k = 1:m;
%runs this master loop for each of the different number of channels
GH = k*1e-16; %the proton conductance is equal to the unitary conductance %times the number of channels
GNa = k*0; %the Na conductance is equal to the unitary conductance %times the number of channels
GCl = k*0; %the Cl conductance is equal to the unitary conductance %times the number of channels
end

for j = 1:2;
%run this inner loop twice for each number of channels
%builds vectors and assigns initial values
EH = zeros(1,n);
EK = zeros(1,n);
ENa = zeros(1,n);
ECI = zeros(1,n);
VM = zeros(1,n);
JH = zeros(1,n);
JK = zeros(1,n);
JNa = zeros(1,n);
JCl = zeros(1,n);
totHi = zeros(1,n+1);
totHo = zeros(1,n+1);
Ki = zeros(1,n+1);
Ko = zeros(1,n+1);
Hi = zeros(1,n+1);
Ho = zeros(1,n+1);
Nai = zeros(1,n+1);
Nao = zeros(1,n+1);
ClI = zeros(1,n+1);
Clo = zeros(1,n+1);
Ki(1) = Ki1;
Ko(1) = Ko1;
Hi(1) = Hil;
Ho(1) = Hol;
Nai(1) = Nail;
Nao(1) = Nao1;
ClI(1) = ClI1;
Clo(1) = Clo1;
totHi(1) = (BKd + Hil + Bi)/(1+(BKd/Hil));
totHo(1) = (BKd + Hol + Bo)/(1+(BKd/Hol));
if j == 1
%changes the value of x for each of the two times %through the loop
x = 1; %x is used as a multiplier of the membrane voltage Vm
else
%depending on the orientation of the channel the %membrane voltage is
x = -1; %either positive or negative, this has a large impact %on open probability
end
for i = 1:n;  %this loop runs the solver for equivalent circuit
differential equation

EH(i) = ((R.*T)./F).*log(Ho(i)./Hi(i));  %calculates the equilibrium potential for H+

EK(i) = ((R.*T)./F).*log(Ko(i)./Ki(i));  %calculates the equilibrium potential for K+

ENa(i) = ((R.*T)./F).*log(Nao(i)./Nai(i));  %calculates the equilibrium potential for Na+

ECl(i) = -(R.*T)./F).*log(Clo(i)./Cli(i));  %calculates the equilibrium potential for Na+

VM(i) = (GH.*EH(i) + GK.*EK(i) + GNa.*ENa(i) + GCl.*ECl(i))./(GH+GK+GNa+GCl);  %calculates the membrane potential

JH(i) = (GH./F).*(VM(i)-EH(i))./(1+exp(-z*F*(x*VM(i)-Vmid)/(R*T))));  %determines flux for H+ mol/s

JK(i) = (GK./F).*(VM(i)-EK(i));  %determines flux for K+ mol/s

JCl(i) = -(GCl./F).*((VM(i)-ECl(i));  %determines flux for Cl- mol/s

JNa(i) = (GNa./F).*((VM(i)-ENa(i));  %determines flux for Na+ mol/s

totHi(i+1) = totHi(i)-(JH(i).*dt)./Vi;  %determines concentration
totHo(i+1) = totHo(i)+(JH(i).*dt)./Vo;  %determines concentration
totHi(i+1) = totHi(i)-(JH(i).*dt)./Vi;  %determines concentration
totHo(i+1) = totHo(i)+(JH(i).*dt)./Vo;  %determines concentration

Ki(i+1) = Ki(i)-(JK(i).*dt)./Vi;  %determines concentration

Ko(i+1) = Ko(i)+(JK(i).*dt)./Vo;  %determines concentration

Nai(i+1) = Nai(i)-(JNa(i).*dt)./Vi;  %determines concentration

Nao(i+1) = Nao(i)+(JNa(i).*dt)./Vo;  %determines concentration

Cli(i+1) = Cli(i)-(JCl(i).*dt)./Vi;  %determines concentration

Clo(i+1) = Clo(i)+(JCl(i).*dt)./Vo;  %determines concentration

Hi(i+1) = (-Bi-totHi(i+1)+BKd)+sqrt((Bi-totHi(i+1)+BKd).^2 + 4.*BKd.*totHi(i+1)))./2;  %determines the free H+ concentration

Ho(i+1) = -((Bo-totHo(i+1)+BKd)+sqrt((Bo-totHo(i+1)+BKd).^2 + 4.*BKd.*totHo(i+1)))./2;  %determines the free H+ concentration

end

sig = (1e-6 - Hi)/(1e-6 - 1e-7);  %calculates the internal pH change

if j == 1  %if the channel is in the slow
%conducting configuration
sflux(k,:) = sig;  %assign phi to sflux of k channels
else
    fflux(k,:) = sig;  %assign phi to fflux of k channels
end
end

time1 = 0:1:n;  %time vector
time1 = time1.*dt;  %standardizes the time to seconds

fflux2 = zeros(m,floor((n+1)/100));
sflux2 = zeros(m,floor((n+1)/100));
time2 = zeros(1,floor((n+1)/100));

for r = 1:floor((n+1)/100);  %data reduction loop
    sflux2(:,r) = sflux(:,r*100);
    fflux2(:,r) = fflux(:,r*100);
    time2(r) = time1(:,r*100);
end

sflux = sflux2;
fflux = fflux2;
time1 = time2;

figure
plot(time1,fflux,time1,sflux)  %plots all 60 flux time courses

pt_ffluxbias  %calls and executes the script pt_ffluxbias (see below)
total_proportional Flux_V  %calls and executes the script total_proportional_flux_V (see below)
function pt_ffluxbias

% builds pascal's triangle *(1/2)^{(number of elements in row)}
% then if there are any channels in the fast fluxing orientation it takes
% the flux vector from fflux for however many channels there are in that
% orientation, if there are only slow fluxing channels it takes the flux vector
% form sflux for that number of slow fluxing vesicles
% in each case it multiplies the flux by the ratio given by the probability
% of that configuration and then sums all of the fluxes for a given total
% number of channels per vesicle

global time1 % retrieves the value of the time vector from simple_flux_V
global fflux % retrieves the value of the fflux matrix from simple_flux_V
global sflux % retrieves the value of the sflux matrix from simple_flux_V
global pflux % assigns the pflux matrix as a global variable

[m n] = size(fflux); % uses the dimensions of fflux to assign value to script
% components

pflux = zeros(m, n); % builds pflux matrix

for k = 1:m % run for each number of channels per vesicle
    % k defines the total number of channels in this run

    cflux = zeros(1, n); % builds cflux vector

    for j = 0:k; % j defines the number of fast flux oriented channels
        f = (factorial(k)/(factorial(j)*factorial(k-j)))*(1/2)^k;
        % f calculates the fraction of vesicles with k channels, j of them
        % in the fast fluxing orientation and (k-j) in the slow fluxing
        % orientation

        if j == 0 % if there are no channels in the fast fluxing orientation
            cflux = cflux + sflux(k,:)*f;
            % assign the flux as slow flux of k channels
        else
            cflux = cflux + fflux(j,:)*f;
            % assign the flux as fast flux for j channels
            % this assumes that the contribution to flux from fast fluxing
            % channels is >> than that from slow fluxing channels
        end
    end
    pflux(k,:) = cflux; % assigns total flux for k number of channels to pflux
end

figure
plot(time1, pflux)
function y = total_proportional_flux_V

global timel            %retrieves the value of the time vector from simple_flux_V
global pflux            %retrieves the value of the pflux matrix from pt_ffluxbias
global totalflux        %assigns the totalflux matrix as a global variable
global rho

[m,n] = size(pflux);   %uses the dimensions of fflux to assign value to script components

cp = 0.1;             %set the vector of protein concentration in vesicle mixtures
%currently set for 1:100 ratio of protein-to-lipid (see channel_dist_theta
%input) but can be set to any/many protein-to-lipid ratios for example see
%the first comment line directly above.

rho = channel_dist_theta(7.57,100,cp);   %determines the Poisson distribution of number of channels per vesicle by
%running the channel_dist function for lipid concentration of 10 mg/ml and
%average vesicle radius of 50 nm

p = length(cp);       %uses the length of cp to assign value to script components

rho2 = rho(:,2:m+1);  %removes empty vesicle component of rho

totalflux = zeros(p,n); %builds totalflux matrix

for j = 1:p;           %for each protein concentration
    fluxp = zeros(m,n); %builds fluxp vector
    rho2(j,:) = rho2(j,:)./sum(rho2(j,:));   %scales each column of rho2 to its total value
    for i=1:n; %for each column of pflux
        fluxp(:,i) = pflux(:,i).*rho2(j,:);  %multiplies dot-wise each column of pflux by the scaled probability
            %for that number of channels per vesicle given the protein to
            %lipid ratio
    end
    totalflux(j,:) = sum(fluxp);       %assigns the total flux for each protein to lipid ratio
end

figure
plot(timel,totalflux)
axis([0 max(timel) -0.05 1.05])
xlabel('Time /s')
ylabel('Scaled Vesicle Flux')
title('Scaled Flux by Vesicles Containing Protein for each Protein:Lipid Ratio')
function y=channel_dist_theta(cl,r,cp)
%cl final concentration lipids
%r radius of vesicles in nm
%cp final concentration of protein

global pflux

[m,n] = size(pflux);

p = length(cp);

massl = cl*(10^-3); %mass in grams per 10 mililiter
%mass = mg/ml*(10^-3 g/mg) = g/ml

numlipid = (massl/753.84)*(6.022e23); %number of lipids in sample
%numlipid = (mass lipids in g/ average MW lipid)*Avogadros number

SAlipid = (numlipid*63e-20)/2;
%total surface area of lipid head group in m^2
%factor of 2 remove for bilayer

Nv = SALipid/(4*pi*(r*1e-9)^2); %calculates the number of vesicles

Np = ((cp*(10^-3))./(2*35000))*6.022e23; %number of channels per ml

f = zeros(p,m);

for i = 0:m;
    f(:,i+1) = ((((Np./Nv).^i).*exp(-Np./(Nv*0.85)))./(((0.85)^i)*factorial(i)));
end

f = f*0.85;

f(:,1) = f(:,1)+0.15;

y = f;

function unscaled_plot(tf,rho0)

global timel
global slow

p = length(rho0);

for i = 1:p
    tf(i,:) = tf(i,:)*(1-rho0(i));
    tf(i,:) = tf(i,:)+rho0(i);
end

slow = tf(p,:);

figure
plot(timel,tf)
axis([0 max(timel) -0.05 1.05])
xlabel('Time /s')
ylabel('Scaled Total Flux')
title('Scaled total Flux for each Protein:Lipid Ratio')
APPENDIX II

Sequences of all putative H\textsubscript{v} channel genes that were synthesized for expression and biochemical stability experiments. Each sequence is named according to its GI number followed by its species. A multiple sequence alignment done using ClustalW of all putative H\textsubscript{v} channel genes is also shown compared to the sequence of the Rat K\textsubscript{v}1.2 voltage-sensor domain (with its transmembrane helices highlighted in cyan). Conserved residues of possible functional importance are highlighted in yellow (with outliers highlighted in alternate colors).

Sequences of Putative H\textsubscript{v} channel Genes

>91992153_Homo_sapiens (Human)
MATWDEKAVTRRAVPAERMKSFLRHTVVDYHAWNNYKKWENEEEEEEEQPPP
TPVSGEEGRAAPDVAPAPGPAPRPLDFRGLKLSRHRQVIIICLVVLDDLVL
ELILDLKIIQPDKNNYAMVHFMSITILVFFMMEIIFKLFFVRFELFHKFEILD
AVVVVFILDDVQEQHEQFEALGLLLHLLLWRLVRVARIIISVKTRSERQLLRLKQM
VQLAAKIQHLEFSCSEKEQIERLNKLRLRQHGLLGEVN

>118344228_Ciona_intestinalis
MEGDNCNKSRRHKSHMINPYNASVRCTQPPLPSVIQLRSRNKMIGTEDPSSDSEP
SSNQPLLTLNSYEVHTFNDNHNRPAPQEQSTQNTMISMQSEQKSDKFTASNLGMF
QMKFEIGEDGDDBEEEAILTNREKLRLHSHKPIHVAILLTVLSDFLVGEILLDLK
IVIVPHGNPAPEILHGFSLISIFMVEIALAIADHRFHIIHKVEVLDADV
VVVISFGVDIALIFVGGESEALAAIGLLVLRLWRVFRIINGIIIVTVKTKADD
RVEHEIKKNESELQIHNLEEKLSQEQDMSRLHEILRCNNIDIPPTVPLTTSQIHST
TASADV

>71897219_Gallus_Gallus (Chicken)
MSRYLKHTTVGDPQWSNDYQKWENEEDNGEKDEIKLEPSRGHVTFQDVMKKS
SSRPFQIVIVFLVIDALLVLGEILMLKIIHPDKYIAPKFHYLSLISITILFLVEVG
FKIFVHGEEFFHKHSINVQLSLVIDLLVFREHEFAVGLLLIRLRWVARII
GIIISVKTSEQQSVSKEQVLKLVKLATKVQHSCVEKEQIERLRLMLKQHGL
SEQT

>6573743_Arabidopsis_thaliana
MNIINTGTVDVFQTNLIKSWCRRRKWRQCNFSPKQQELISINQWRITLNSNL
ESYQVHLFTIPLSLDILTSLLELSSSSLSCSTVKTETENWFRGWGTVILS
LAVKSMALVVAAGKSFQPGCCMDGTLAIVALLQVLLEKKGTFIVVSVLWR
LVRVETAFE
LSDEAIIEVQIDGIISQFQASLMKRLSTELTAKEKDEVKLMEEENRFKENG
DIPFVKP
>116505721_Coprinopsis_cinerea
MPLQIFIDVVFVLVELGYLFNPNCELEPEPRTVPWMEALSTSLALSLALLVTETPITV
WCMGIQYPVNGAVWAALHFLDALINLATFILDLVLRGERELASLLILRRLRIAKL
VSSVAVATDSLEEEVEAREATQELHRTKEELGKVEEEEVFNLRQLATFETKVSNSA
V

>329664616_Bos_taurus_(Cow)
MATWNEKAVTRRARVAPAERMSKFLKHFTRVGDYHAWNYKKWENEDEEEEEEQPPPT
EASASAEERATDPTAPAPVPRPRPLDFRTRTLRKLFFSHRFQVIIICLVLDDLALLLAE
LVLDLKIIEPDKNYAPKVFHYSMSLAILTFFMEIFFKIFVFRLEFFHKFEILDTIVV
VISFILDIVLFFREHQQEALGLLILLRLRLWVARINGIISVKTIIQQLQLRQINIQ
LATKIQHLESCSEKEQEIERLKNLRLRQHQLGGEV

>345790859_Canis_lupis_familiaris_(Dog)
MATWDEKAASSRRAVAPAERMSKFLKHFTRVGDYHAWNYKKWENEEDDEEEEEQPPPP
TAASGEERADPTAAPTRPLEDFRATLRKLFFSHRFQVIIICLVLDDLALLLAEILILD
LKIQGDKNYATKVFLHYSSFAILTLFMMEVFLKLFVFRLFFHHKFEILDTFVVVSF
ILDLVLLFFKHEFALGLLLILLRLRLWVARINGIISVKTIIQQLQLRQINIQ
LATKIQHLESCSEKEQEIERLKNLRLRQHQLGGEV

>148235789_Xenopus_laevis_(Frog)
MAGCLRHFTSVGDDTKKREWQEDVEVAYEELKNTPHFIAAYSFRGALKWLLSSHKF
QIVIICLVLDDLAFVLVEVLDDLLELAEKVHIIPEFHYLSISVTTFILEAIAGKLYA
FRLEFPFHHKFEVFDAAIVVISFIIDIVVISREDIFNAVGLLLILLRLRLWVARINGIV
VSVKTRAEEKMKLKEQKGSLEKEQALEQCPQQEIEGRHLKLLQEHNFV

>83774308_Aaspergillus_oryzae
MASPSDPLLHEHTGPRSLRQFIYLPEDQEQQRIIAWRAARDFLSRSSRSGHYLVLLLV
VDVACTFADFLIELHVCELTKGHSHVAIGWGVTVQKVALAVGLVSCLFMLELMVTVSF
GKGYFSKSHVFDALVIIAFAGVDFVALHGIEEELGSLLIVVLRLWVFVIIIEELQSANED
TLEYEHEIERLRQENTYLRQRLVSNLADPM

>124360845_Medicago_truncatula
MIRLVSILLTDIILELSSLVSCKQKVINIEELYFHGIGLISMIKALL
VGLGSFSSKHPGVPVDGIVAILIMEVFLERKGGGLVVSSLVLWVARIVGacesFED
AIEAQIEGIVCFQFAALKDENRLLGIINEDKMLIEKIIKDKC

>109098724_Maca_mulatta_(Indian_Monkey)
MATWDEAKTRRVARAEPMSKFLKHFTRVGDYHAWNYKKWENEDEEEEEEQPPPP
TPASGEERAVAGPAAPAPRALDFRTLRKLFFSHRFQVIIICLVLDDLALLLAE
ELILDLRIIPDQKKNYAMIFHYMSIALALFMMEITFKFLVFRLFFHHKFEILDADV
VVVSFLVDVLLFFQHEFEALGLLLILLRLRLWVARINGIISVKTIIQQLQLRQINIQ
QLAAKIQHLESCSEKEQEIERLKNLRLRQHQLGGEV
>334327101_Monodelphis_domesticus_(opossum)
MGPKQWNNNSGSHTSGQGQTESEQHRQWVPLKDGSPRAMSRFLHFTVGVDDHYKW
TRYYKWDEDEDDEQPVQPTGAPAGTVPGTSESNAVQVPGTVEPTPPAKPDFTVMRLF
GSHRFQVIIICLVMDALLVLAEMLDLKIIPQDKDNAYAVRHVFYLSIAITTFMIEV
ALKYVFREFFYHFKEIFLDADVIIISFVLDIVLLEQEAHEALGGLLILRLWVARIIING
IIISVKTRESQLRKLINHQLATKIQHLESCTEKQEIERLNKLRRDHGLE

>109809754_Mus_musculus_(Mouse)
MTSHDPKAVTRRTKVAPTRKMSFLKHFHVTTVGVDDYHTWNVNYKKWNEEEEEEPA
PTSAEGENAEPDAEAGSATSTRPSLDRFSLRLKLFSSHRFQVIIICLVMDALLVLE
DLKIIPDEQDYAVTAHYSFAILVFFMELIIFFKFVRFLEFFHHKKEILDAFVVVVS
FVLDLVLFLKSHHFALGLLLILRLWVARIIINGIIISVKTRESQILRKLQI

>109497399_Rattus_norvegicus_(Rat)
MRGDTHARGNRLGKGLEAUNAGRMAKQGEAVTRRTKVAPTRKMSFLKHFHVTTVGV
DDYHTWNVNYKKWNEEEEEEPAPTSAEGENAEPDAEAGSATSTRPSLDRFSLRLKLFSS
HRFQVIIICLVMDALLVLEDLKIIPDEQDYAVTAYHYSFAILVFFMELIIFFKFVRFLE
FFHHKKEILDAFVVVVSFVLDLVLFLKSHHFALGLLLILRLWVARIIINGIIIS
VKTRESQILRKLQINIQLATKIQHLESCSEKEQIERLNKLQLQGLGDVN

>187282419_Strongylocentrotus_purpuratus_(sea_urchin)
MFGFRRLSDTTPSEGNDQQRVKVKDSSDSVSVSHDGHPARTEPLSLREKLIHE
MTQKFHIALIVLVIDCILVIVELVIDFELVLSQEEGQCNATETDKEKEKEVTAANV
LYISGISIFMELIILKIPvFREFFRSKLEVFDGIISIVSVLDVSYIYEQQFALQLVL
LRLWHRVVRVNVGVLVLSVEYQAQQKIEQKHRLAEEHEHEKFRYCAAQKEIE)
VLNRTLNQHGIQIDDDYVAKKPQSDLQNLQVNMISADKHDTGEDEEGEGG
TTTREALGTITLTTDDNVNTIQADYHPQDITTFT

>50539752_Danio_rerio_(zebra_fish)
MSRYLKHFTAVGDNSAVFPTWHEEDEVSHVTHLDAPDGLEVSQGQLQLSFRDSLRK
LSTYRFQIVVVLVLDAIFVLELDLISIEADHRHRAIPQVFHYSLALLTTFMMVE
LACKIFAYRELFHLHKFEKDIFVGRVVVSFILDIYISKEDAFAWGMLLILRLWVAR
IINGILVSVQNRANRHEKLMKELKINESLHVSQVNELKEQTONMDQENVRLRALLK
SHSIDF

>156364735_Nematostella_vectorisea_anemone
MESDNQQLVGLVHDFQSLGERAMDKEIEAVEVGDGGGDQVVSSTPCWHILKDRPRLCE
IIHNGKQAQYTIALVIIDCIIVIAELVLDEILKVKVNHPAPILIHSIAILSLIIIE
LIVKIYAMGEMHHKLEEVDIFVSDIALFSGNAAGASILIIIRLWVTRIVNGII
LVSQMKDEKKIHHLKVEELQEEIDLRKTRNÆLENELKTLKGTKEEPVAEEAT

>156374277_Nematostella_vectorisea_anemone_baby
MAEKEVESQTAEDGRKQRTFQONLRRRRSTKEWMQCGCGLRTSLKMLTGQTFIIIL
LVLVEVAINVLVMCISLNAINDSEQHFAZHLHVFGISILAFPALEVFLKFLAGLIEF
KIEKLEIFDAVIVITALIVEILLSAHSTSKAOWKLFVIGNLWVRCVITNIIEFREE
LYELIDESDRSKRTSSATETLHTERESLHETK
>338727680_Equus_caballus_(Horse)
MRGASGTEDTKLSAVLGNCYKDTETTVTRPKVAPERMKLRHFTVGVDDYHTWNINYKKWENEEEEEEOAPPAPASGEGRAEPTAARVPAPRPPPLDFRTLRLKFSCHRFOVIIICLVIDALLVLALIELLDKIEADKNNYVPRVFHYMSLAILTFFMTEVSLKIFVFRLEFFHKFEILDAVVVVVSFVLIDIVLIFREHEFEALGLILLRLWRVARIINGIIISVKTRSERQILRLKQMNIQLAARKVQHFLEFSCSEKEQEIERLNKLLQHGLLGEEVI

>345305006_Ornithorhynchus_anatinus_(Platypus)
MSTPHLGAFRHLLRFVEVSSRASGLRGLRLVIGDQNSRVTIVCVDALLVLAELLLDLRIHAPDEKQVAPKVFHLSDICLTFFVVEVVLKMFMVRLEFFHKFEVLDAVVIISSFIDLVILLFREHEFEALGLILLRLWRVARIINGIIISVKTRSEQQLSRLQANLQLVAKVQHLEFSCNEKEQEIERLNALLKQHGLIN

>156059386_Sclerotinia_sclerotiorum_(Fungal_pathogen)
MSRRNSDISEHAPLIRASSQPISITSELPLYHTPRLSFSRLNGYKSRBVSRLFLSTRGQHYTVLTLVACDLIGIFADIIINLYQCNDKEGKTDPIWNEVVRGIALGFVSCLFMLELISAVWAFGWSKHCFFDATVIVAGFVVDVLLHIGVEEVASLVLIVLRLWRFFKIIEEFSVGAQEQMDVLEERIEQLEMENKRLKELLRKNDNDDELENGERT

>145234953_Aspergillus_niger
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ClustalW Alignment

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<th>Sequence</th>
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<tr>
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192
Aspergillus

Fungal_pathogen

Platypus

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sea_anemone_baby

sea_anemone

zebra_fish

sea_urchin

Rat

opossum

Indian_Monkey

Medicago

Frog

Dog

Cow

Coprinopsis

Arabidopsis

Chicken

Human

rKv1.2

ARIA

91992153_Human

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71897219_Chicken

6573743_Arabidopsis

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109809754_Mouse

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334327101_opossum

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156059386_Fungal_pathogen

345305006_Platypus

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REFERENCES


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Venters, R.A. *et al*., 1996. Characterizing the use of perdeuteration in NMR studies of large proteins: \(^{13}\)C, \(^{15}\)N and \(^1\)H assignments of human carbonic anhydrase II. *Journal of Molecular Biology*, 264(5), pp.1101–1116.


