Properties of Conductance and Inhibition of Proton Channels: M2 from Influenza A Virus and Fo from Escherichia coli ATP Synthase

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PROPERTIES OF CONDUCTANCE AND INHIBITION OF PROTON CHANNELS: M2 FROM INFLUENZA A VIRUS AND F₀ FROM ESCHERICHIA COLI ATP SYNTHASE.

by

J. Craig Moffat

A Thesis submitted to the faculty of

Brigham Young University

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Physiology and Developmental Biology

Brigham Young University

August 2006
This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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Date                      David D. Busath

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Date                      Steven W. Graves
As chair of the candidates graduate committee, I have read the thesis of J. Craig Moffat in its final form and have found that (1) its format, citations and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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Accepted for the College

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Dean, College of Biology and Agriculture
ABSTRACT

PROPERTIES OF CONDUCTANCE AND INHIBITION OF PROTON CHANNELS: M2 FROM INFLUENZA A VIRUS AND F0 FROM ESCHERICHIA COLI ATP SYNTHASE.

J. Craig Moffat

Department of Physiology and Developmental Biology

Master of Science

Proton channels are essential for many of the processes of life. The influenza A viral protein M2 is responsible for sensing the conditions necessary for viral RNA release. The proton-translocating F0F1 ATPase (ATP synthase) uses a proton gradient to drive adenosine triphosphate (ATP) synthesis. We have directly measured proton uptake in vesicles containing reconstituted M2 or F0 by monitoring external pH after addition of valinomycin to vesicles with 100-fold diluted external [K⁺]. This proton flux assay was utilized to quantify proton flux through single M2 and F0 channels.

Contrary to previous reports, proton uptake by M2 was not significantly altered by acidification of the extravesicular pH. We conclude that pH only weakly affects
proton flux through M2 in the pH range of 5.4 - 7.0. Theoretical analysis utilized for such vesicle uptake assays illuminates the appropriate time scale of the initial slope and an important limitation that must be placed on inferences about channel ion selectivity. The rise in pH over 10 seconds after ionophore addition yielded time-averaged single channel conductances of 0.35±0.2 aS and 0.72±0.4 aS at pH 5.4 and 7.0 respectively. Such a low time-average conductance implies that M2 is only conductive $10^{-6}$-$10^{-4}$ of the time. M2 selectivity for hydrogen over potassium is $\sim 10^7$.

$F_O$ translocates protons across membranes, converting electrochemical energy to rotational inertia. Previous experiments have been partially confounded by a contaminating channel, $C_L$, which co-purifies with $F_O$ and leaks cations. $C_L$ activity is shown to not decrease following deletion of the previously uncharacterized $yraM$ open reading frame of $E. coli$. $F_O$ purified from a deletion strain lacking $yraM$ is just as active as $F_O$ purified from the wild-type strain. Using $F_O$ from the deletion strain, the single-hit hypothesis of DCCD inhibition of passive proton flux through $F_O$ was examined. A DCCD-induced reduction in ATP synthase activity correlates with a reduction in the total initial slope, the number of functional $F_O$ per $\mu$g protein, and the single channel proton flux. At least 2 DCCD per $F_O$ are required to totally inactivate passive proton flux. M2 and $F_O$ have similar single channel conductances but different open probabilities.
DEDICATION

There are many individuals who have played a role in my success at Brigham Young University. This work would have never been completed without the loving support of my parents, Jeff and Barbara Moffat. They taught me at an early age to value education, learning, and hard work. This work is also dedicated to my brother Josh. Throughout our lives we have enjoyed a (usually) friendly spirit of competition that has driven both of us to relentlessly pursue our dreams, him to an international fighter pilot program with the United States Air Force and me to science and medicine. May the Lord, who guides and supports all good causes, continue to bless our efforts.
ACKNOWLEDGMENTS

This work would never have been accomplished without the mutual collaborations of Dr. Dixon Woodbury and Dr. David Busath. As an undergraduate I was mentored as a volunteer in their laboratories studying the $F_O$ and M2 proton channels. Attending my first Biophysics conference with them opened my eyes to new possibilities. As a graduate I learned many techniques and gained scientific scholarship from my associations with them. Thank you, both of you.

There are many others to whom I owe a great debt. I thank Dr. Sterling Sudweeks and Dr. Stephen Graves for their support and tutelage as members of my committee. Dr. William Brusilow, with whom I did a rotation, has been an excellent tutor in the world of ATPases. Additionally, he provided numerous samples $F_O$ for our use. Without his collaboration the DCCD studies would not have succeeded. Dr. Philip Gao has gone to great lengths to provide the M2 samples utilized in these studies. Finally, I thank Dr. Viksita Vijayvergiya and the many other researchers in the Great Lab. Without their hard work and patience with me I would still be running experiments!
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Chapter 1 – Introduction and Review of Pertinent Literature

Proton channels and pumps are ubiquitous in nature and play a crucial role in maintaining homeostasis. A review of all mechanisms of proton translocation through lipid membranes is not feasible in this work. The interested reader should see DeCoursey’s excellent and extensive review of that topic (1). Uses of proton channels and pumps range from bacterial channels that guard against acidic conditions; mammalian pumps that acidify the stomach via a H⁺-K⁺ antiporter; eukaryotic and prokaryotic ATP synthase that uses a hydrogen electrochemical gradient to convert ADP and phosphate to ATP; and an influenza viral proton channel that triggers viral uncoating after cellular infection. This report will focus on the M2 proton channel from Influenza A virus and the F₁F₀ ATPase (ATP synthase) from *E. coli*.

**M2 Proton Channel from Influenza A Virus**

The Influenza A life cycle is summarized in Figure 1-1. Hay (2) and Grambas (3) have described the viral life cycle. The virus adheres to the host cell using hemagglutinin (HA), found in the viral membrane, to bind sialic acid, one of the carbohydrates found on the host cell membrane (4). The virus is then encapsulated into an endosome. The low pH triggers M2 activity, acidifying the interior of the virus, which facilitates viral fusion to the endosome and release of ssRNA into the cellular matrix. After RNA replication and transcription, the virus forms by budding from the cellular membrane, perhaps from lipid rafts where the neuraminidase and hemagglutinin have been shown to localize (5,6). Neuraminidase cleaves sialic acid from the polysaccharide structure of the host cell’s membrane, releasing the newly
formed virus (4,7,8). Amantadine has been shown to block M2 activity, inhibiting viral synthesis by preventing viral acidification and therefore RNA release. Another class of anti-viral drugs that target the flu virus is called neuraminidase inhibitors, which act to prevent viral budding and to cause clumping of free viral particles. In some situations, M2 is thought to prevent acidification of the golgi apparatus to protect acid-sensitive hemagglutinins.

M2 is useful to study for two reasons. First, the simplicity of the channel relative to other more complex proton channels, such as $F_O$, make M2 a useful model to study. The active form of M2 consists of a homotetramer (9,10). Figure 1-2 is a molecular model of the M2 tetramer showing the protein backbone and histidine-37, which are implicated as the proton selectivity filter. There is evidence that M2 is more permeable to protons at lower pH (3), a process termed acid-gating.

Second, the mechanism of action of amantadine is of interest because although it has been available by prescription since 1987 to treat flu (type A), the mechanism of inhibition has not been fully clarified. In Figure 1-2 amantadine has been overlaid in a space-filling representation near the selectivity pore (as simulated by Mario Pinoli). Although its exact M2 binding location is unknown, it may be an intrachannel (11,12) or an allosteric blocker (13). This figure shows that if amantadine is able to reach histidine-37, it is not likely to pass through due to steric hindrance. Greater understanding of amantadine activity may lead to more effective M2 blockers and perhaps better anti-viral drugs.
**F_1F_0 ATPase from E. coli**

Proton transport is also a key step in the production of ATP by the F_1F_0 ATPase. ATP synthase is vital for life in organisms from both the plant and animal kingdoms. It catalyzes the last step in oxidative metabolic pathways using an electrochemical gradient to make ATP from ADP and phosphate. It is noteworthy that this mechanism can run in the reverse direction so that ATP hydrolysis can be used to pump protons up an electrochemical gradient to acidify compartments within cells or organs.

Figure 1-3 shows the structure of ATP synthase. F_0 is the proton transport sector that is embedded in the membrane. Researchers have demonstrated that F_0 rotates in the plane of the membrane (14). It is composed of a ring of c subunits, two b subunits, and one a subunit. The exact number of c subunits has been debated in the literature for decades (summarized in Table 1-1). Knowing this number is critical to determine how many protons are required for each ATP generation.

The F_1 sector of the F_1F_0 ATPase is the site of ATP formation or hydrolysis. It is composed of three sets of α and β subunit pairs and one each of γ, δ, and ε subunits. The ATP is formed within the αβ pairs by the rotation of the γ subunit such that 3 ATPs are produced (or hydrolyzed) each full rotation. The γ subunit is linked to the c subunit ring, which rotates in the membrane based on the electrochemical proton gradient that is created by metabolic pathways.

Mechanically, the F_0F_1 ATPase is interesting to study because it is a true motor. Like an electrical generator, ATP synthase has a stator (a, b and δ subunits) and a rotor (c ring, γ and ε) (15). Rotation of c with respect to a and b is then
transferred to the stalk (γ and ε) producing conformational changes in α and β to make ATP. ATP synthase represents one of the smallest true biological motors. Researchers have quantified the rate of proton flux through F₀. Although estimates vary by more than a factor of 1,000 (70 H⁺/F₀/sec (16), 10⁵ H⁺/F₀/sec (17)), the two most recent publications estimate the flux to be 6240 protons per second per F₀ (18) and 3100 protons per second per F₀ (19).

\(N,N'\)Dicyclohexylcarbodiimide (DCCD) has been shown to inhibit F₀ function by covalently binding to aspartate-61 on the c subunit of F₀ to form an N-acyl urea (20). Thus DCCD selectively blocks F₀, presumably inhibiting proton flux by making the proton binding site unavailable. Since there are many c subunits per F₀, the number of DCCD required to block proton flux per F₀ has been investigated but remains unclear. As further described in Chapter 3, there is evidence in support that modification of just one c subunit by DCCD will block transport (single-hit model). There is also evidence in support that more than one c subunit must be “hit” by DCCD to block transport (multiple-hit model).

**Specific Aims**

There are two main aims of my master’s thesis:

1. Examine the proposed acid gating of M2
2. Examine the proposed single-hit stoichiometry of DCCD inhibition of F₀.

A significant obstacle for Aim 1 was designing comparable buffers at different pHs in which to run the experiments. The four histidines at position 37 of M2 have been shown to have pKₐ values of 8.2, 8.2, ~5.7, and <4. It seemed most useful to study pHs above 8.0, between 8.0 and 5.7, and below 5.7 (21). Simulations by Dr.
Busath and Steve Later suggested that the proton flux assay results were very sensitive to the pH at which the experiment was run in relation to the pK$_a$ of the buffer in which the experiment was run. For reliable results they suggested that the studies be performed 0.2 pH units below the pK$_a$ of the weak acid used to buffer the extra- and intra-vesicular pHs, confirming an earlier hypothesis.

Design of the three buffer systems for pHs 5.4, 7.0, and 9.4 was accomplished, as reported in Chapter 2. However, 75% of all experiments at pH 9.4 failed to give any signal, perhaps due to the buffer glycine permeating the vesicles. At the other two pHs no evidence of acid gating was found. In a separate experiment, gramicidin was incorporated into vesicles as a positive control for leakiness to K$^+$. This experiment proved useful in strongly demonstrating that perfect selectivity for protons against potassium is not essential for the proton flux assay to work. As discussed in Chapter 2, a gramicidin dose response curve revealed that if too much of a mildly or weakly selective channel is added to the vesicles the proton flux assay will fail. Chapter 2 also presents a detailed mathematical model of the proton flux assay, as modeled by Dr. Busath. He contributed much of the discussion of that chapter which is being submitted for publication.

In regards to Aim 2, examining the proposed single-hit stoichiometry of DCCD inhibition of F$_0$, there are three possible results. First, Hermolin and Fillingame (22) concluded that one DCCD molecule inactivates ATPase activity, which they called the single-hit model. The single-hit model of ATPase inactivation is often assumed to apply to passive proton translocation as well. However, previous research by Kopecky et al. (23) and Glaser et al. (24) showed that while one DCCD
per F\textsubscript{0}F\textsubscript{1} ATPase was sufficient to maximally inhibit ATPase function, two DCCD per F\textsubscript{0} were required to maximally inhibit passive proton flux, with a reduced block occurring at 1 DCCD per F\textsubscript{0} (single-hit concentration). More recently Dmirtiev et al. (25) attempted to show that mutation of one Asp61 is sufficient to inhibit F\textsubscript{0} activity. Incorporation of one c subunit with an Asp61Gly mutation appears to inhibit F\textsubscript{0} activity. However, mutation of Asp61 to the structurally similar but non-protonatable Asn failed to show such potency.

As discussed further in Chapter 3, our assay will be able to distinguish between the single-hit and the multiple-hit hypotheses. Using F\textsubscript{0} that is purified after partial inhibition of ATPase activity by DCCD, we will determine extent of inhibition of passive proton flux. By comparing the proton flux inhibition to the inhibition of ATPase activity we can distinguish between the models in the following manner. If the single hit hypothesis is correct, the expected result from the proton flux assay would be: the single channel flux (H\textsuperscript{+}/F\textsubscript{0}/sec) remains unchanged, the number of functional F\textsubscript{0} is reduced, and the initial slope (H\textsuperscript{+}/sec) is reduced. If the second hypothesis is correct, i.e. it requires 2 (or more) DCCD/F\textsubscript{0} to inhibit passive proton flux but 1 DCCD/F\textsubscript{0} to inhibit ATPase activity, we will observe a reduction in the single channel flux (H\textsuperscript{+}/F\textsubscript{0}/sec), an unchanged number of functional F\textsubscript{0}’s, but still a reduction in the initial flux (H\textsuperscript{+}/sec).

**General Methods**

**Proton Flux Assay**

In order to accomplish Aims 1 and 2, I will need to be able to measure the number of protons that go through individual channels. The proton flux assay was
adapted to study $F_0$ by the Woodbury lab as described in Franklin et al (19), Dr. Franklin’s dissertation, and Chapter 3. This assay has been adapted in Dr. Busath’s lab to study M2, as detailed in Chapter 2. The basic idea of the proton flux assay is summarized in Chapter 2 and as follows. Dr. Brusilow or Dr. Gao both ship purified $F_0$ or M2, respectively, to our lab. The protein is then incorporated into vesicles with high concentrations of intravesicular potassium and buffer. A pH probe records changes in pH of the weakly buffered external solution during the following steps:

1. The extravesicular solution is continuously stirred and the pH monitored. After the extravesicular pH stabilizes, vesicles are added.

2. Valinomycin, a potassium transporter, is added to the solution, allowing potassium to diffuse out of the vesicles down its concentrations gradient. This creates a net negative charge in the vesicles slowing the efflux and drawing protons into the vesicles through functional proton channels. This is detected by the pH probe as basification of the extravesicular solution.

3. CCCP, a proton transporter, is added allowing any vesicles without functional proton channels to equilibrate their negative charge. This acts as a positive control for the presence of vesicles and as a way to check the incorporation of functional channels into our vesicles.

4. The buffer strength of the extravesicular solution is then determined by back-titrating the solution with one or more additions of HCl.

To observe drug sensitivity of the proton channels, the appropriate blocker is preincubated with vesicles during or before step 1.
Analysis of the data is performed using several customized Excel spreadsheets I created. The raw data is obtained from the probe, which is in volts. This is plotted versus time. The slope of the valinomycin signal over 10 seconds is fitted to a straight line. The average height of the back-titrations is then used to convert the slope (in volts/sec) to moles H⁺/sec. This is then divided by the number of vesicles, which is estimated to be 4 x 10¹¹ based on an assumed diameter of 250 nm, 9 mg/ml lipid, and 63 Å² for the area of one lipid head group. For actual analysis of experiments performed in these studies the true size of each batch of vesicles was determined using Dynamic Light Scattering (DLS) after vesicle sonication or extrusion through a sizing filter. The methods for the individual studies are more carefully detailed in Chapters 2 and 3.

The Fₐ studies were performed under Dr. Dixon Woodbury’s guidance. The M₂ studies were performed under Dr. David Busath’s guidance. Dr. William Brusilow, Professor of Biochemistry at Wayne State University School of Medicine, is an expert in ATP synthase and a longtime collaborator with Dr. Woodbury and performed the yraM deletion, the ATPase inhibition and Fₐ extraction techniques (as described in Chapter 3). I did a two-week rotation in his laboratory during the summer of 2005, learning biochemical techniques associated with Fₐ purification. Dr. Philip Gao is a longtime collaborator with Dr. Busath and provided the M₂.
Reference List


protein, possesses an apical sorting signal in polarized MDCK cells.


National Academy of Sciences of the United States of America.

101:12159-12164.
Figure 1-1 – Influenza A virus life cycle taken from (26) with emphasis on the roles of M2, hemagglutinin, and neuraminidase. M2 plays a key role in recognizing the cellular pH environment and triggering fusion of the virus capsid to the endosomal membrane when an acidification is detected.
**Figure 1-2** – Model of M2 structure by Mario Pinoli with histidine 37 shown in a ball and stick model. Amantadine (space filling model) has been superimposed on the image to emphasize the constriction created by the four histidines.
Figure 1-3 – Structure of $F_0F_1$ ATPase as reported in Aksimentiev (27). The $F_0$ sector is the membrane bound portion that translocates protons. $F_1$ is the cytoplasmic domain that functions either as an ATPase or ATP synthase. $F_0$ rotation drives ATP synthesis. Conversely, ATP hydrolysis can drive proton flux through $F_0$. 
Table 1-1 – Summary of the reported number of c subunits per $F_O$ sector. There is still uncertainty in the number as various groups continue to report different answers under different conditions. It has been suggested that the actual number of c subunits in a newly produced $F_O$ may be varied by a cell in response to different environmental conditions.

<table>
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<tr>
<td>8-14</td>
<td>(28,29)</td>
<td>1982, 1989</td>
</tr>
<tr>
<td>12</td>
<td>(30)</td>
<td>1998</td>
</tr>
<tr>
<td>10</td>
<td>(31)</td>
<td>1999</td>
</tr>
<tr>
<td>14</td>
<td>(32)</td>
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<tr>
<td>11</td>
<td>(33,34)</td>
<td>2003, 2001</td>
</tr>
<tr>
<td>10</td>
<td>(35)</td>
<td>2004</td>
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Chapter 2 - Direct Measurement of Proton Transport Through Influenza Virus A M2 Protein Reconstituted in Vesicles

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Keywords: Proton uptake, Donnan equilibrium, amantadine, single channel conductance, lipid membranes, channel selectivity
Abstract

Influenza A virus M2 protein is known to form acid-activated, proton-selective, amantadine-sensitive channels. We have directly measured proton uptake in vesicles containing reconstituted M2 by monitoring external pH after addition of valinomycin to vesicles with 100-fold diluted external $[K^+]$. Proton uptake was not significantly altered by acidification. Under neutral conditions, external addition of 1 mM amantadine produced a reduction in proton flux consistent with randomly ordered channels. Amantadine block was reduced at pH 5.4. In accordance with a previous study of reconstituted M2 using a pH sensitive dye to monitor intravesicular pH (1), we conclude that bath pH weakly or does not significantly affect proton flow in the pH range of 5.4 - 7.0 for the reconstituted system, contrary to results from electrophysiological studies. Theoretical analysis of the relaxation to Donnan equilibrium utilized for such vesicle uptake assays illuminates the appropriate time scale of the initial slope and an important limitation that must be placed on inferences about channel ion selectivity. The rise in pH over 10 seconds after ionophore addition yielded time-averaged single channel conductances of $0.35 \pm 0.20 \text{ aS}$ and $0.72 \pm 0.42 \text{ aS}$ at pH 5.4 and 7.0 respectively, an order of magnitude lower than was previously reported in vesicles. Assuming complete membrane incorporation and tetramerization of the reconstituted protein, such a low time-average conductance in the face of the previously observed single channel conductance (6 pS at pH 3) implies a channel $Po$ of $10^{-6}-10^{-4}$. M2 is $\sim 10^7$ selective for hydrogen over potassium.
Introduction

The influenza virus M2 protein, target of the antiviral drugs, amantadine and rimantadine, forms an acid-activated proton-conducting ion channel which functions during uncoating and maturation by modifying the pH in virions as well as in trans-Golgi vesicles (2,3). The M2 channel is known to be highly selective for protons and has low permeability for other physiological ions according to reversal potential studies (4). The ion channel activity has been observed in whole cell *Xenopus* oocytes, mammalian cells, and yeast cells (2,4,5,6,7,8) and in planar lipid bilayers (9). This protein is a homotetramer of 97 amino acid (10,11) residues with 23 amino acids of the N-terminus oriented extracellularly, a single internal hydrophobic domain of 19 residues that acts as a transmembrane domain and forms the pore of channel, and a 54 residue cytoplasmic tail. Histidine37 (His37) within the transmembrane domain has been implicated in the activation and proton selectivity of the channel and may be involved in proton translocation (8). Tryptophan41 (Trp41) has been shown to influence the pH dependent characteristics of the channel (12).

The shape of the channel has been well characterized. M2 is a symmetric or pseudosymmetric tetramer (13) with the membrane spanning region being a left-handed coiled coil (14,15). The helices are separated by 8 Å and the Trp41 on the \(i^{th}\) helix and His37 of the \(i^{th} + 1\) helix are paired, being separated by only 3.9Å (16). The helical tilt pivots near His37 and is flexible to allow the membrane spanning region to fit within the bilayer thickness (17). This accounts for the variety of tilt angles from the bilayer normal of 15° to 38° reported in a variety of lipid systems (14,17,18,19,20). Using hydrogen/deuterium exchange with the whole protein Tian et
al. (21) showed the presence of an aqueous pore. These data support the notion that M2 homotetramers form an ion conduction pathway.

The mechanisms of selective M2 proton conductivity (22) and pH activation (23) are matters of current debate. Two main selective conductivity mechanisms have been suggested: gated Grotthus conductance (24) and shuttling (25). In the gated Grotthus mechanism, conductivity is achieved when water molecules are able to penetrate the channel throughout, forming a continuous, conductive proton wire. In the shuttling mechanism, the histidines are directly involved in the proton transfer mechanism. A biprotonated histidine intermediate is transiently formed, leading to rapid proton release at the opposite side of the histidine ring. Regeneration occurs through tautomerization or flipping of the imidazole ring. A detailed kinetic analysis of the M2 current, which fits either mechanism, has been presented by Lear (26). An intermediate mechanism involving water molecules on either side of the imidazole rings serving as the shuttling agent has been suggested based on simulations (27). Details of the protein structure and dynamics underlying selective proton transport are still far from settled.

Conceptually, one would expect the term “acid-activation” to refer to increased flux protons at lower pH, above and beyond what one would expects from mass-action (28), modified by saturation (4); or, to increased probability of the open state (Po) for single channels. Although proton current does go up by 2-10 fold as pH is reduced by 1.5-2 pH units (2,4,6,28,29,30,31) the increase is generally an order of magnitude lower than expected from mass-action, let alone with acid-gating in addition to mass-action. The increase is even lower in the one previously reported reconstitution
experiment (1), where a pH change of 1.4 pH units induced only a 2-fold increase in vesicle proton uptake. These sub-mass-action increases could be due to saturation of an obligatory site in the proton transport pathway. Clear experimental evidence of acid-activation can be observed when extracellular fluid is basified: whole cell outward M2 current is decreased, even though the driving force on protons is increased (4). For reconstituted M2, where single channel currents have been observed, single channel currents increase with decreasing pH as expected from mass-action (modulated by saturation, (9)), but Po changes have not been determined, so no evidence of acid-gating is yet available at the single channel level.

Proton translocation into vesicles with reconstituted M2 proteins has been measured previously using an intravesicular pH sensitive dye (1,32). Here, we report the direct measurement of extra-vesicular pH changes associated with the permeability of M2 in vesicles. We used the pH electrode method reported earlier (33,34) to examine the effects of amantadine and variation in pH. The protein channel activity was monitored by measuring the change in pH of the weakly buffered external solutions. As with previous studies, determining the number of functional M2 proteins in our assay was difficult because of the possibility of variable incorporation, variable oligomerization (or non-functional incorporation), and non-uniform partitioning of the protein into the liposomal membranes. In this study, we have determined the average proton flux per vesicle. Dividing by the nominal number of M2 tetramers per vesicle we determine the time-averaged single channel conductance of M2. Some of the results have been presented previously in preliminary form (35,36,37).
Materials and Methods

Purification and Reconstitution of M2 from E. coli.

M2 protein was expressed and purified using previously published methods (38). Briefly, the M2 protein, Udorn variety with a six-His tag at the C-terminus and serine substitutions for C19 and C50 was expressed in BL21 (DE3) cells using the PET 39 plasmid and purified from exclusion bodies with a Ni affinity column or using DEAE. Gel electrophoresis revealed the presence of single band in a sodium dodecyl sulphate gel (Figure 2-1). Sequence variants containing a His-tag at the N-terminal, His-tag cleaved with Tobacco Etch Virus (TEV) protease, or His37 mutated to alanine (H37A) were also expressed and purified similarly and used for the study.

Protein concentration was determined using the bicinchoninic acid method. The protein was reconstituted into 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-sn-glycero-3-[phosphoro-rac-(1-glycerol)] (DMPG) lipids (4:1 molar ratio; Avanti Polar Lipids, Alabaster, AL) at a 1:5 protein:lipid (w:w) ratio using 1% n-octyl β-D-glucopyranoside (O.G.) and then dialyzed three times. The dialyzed sample was centrifuged and resuspended as proteoliposomes in aqueous solution.

Reconstitution of M2 protein in liposomes.

This step involved two procedures. First protein-free lipid vesicles were prepared and then these vesicles were mixed with M2 proteoliposomes by freeze thaw sonication. The size of the resulting liposomes was measured by dynamic light scattering (DLS, 90Plus Particle Size Analyzer, Brookhaven Instruments Corporation,
Holtsville, NY). For experiments at pH 7 and pH 5 different intravesicular and extravesicular buffers were used.

**Preparation of Lipid Vesicles.**

Stock solutions of L-6-phosphatidlyethanolamine (PE), L-α-phosphatidylcholine (PC), L-6-phosphatidylserine (PS) from brain, and cholesterol each at 10 mg/ml in chloroform were mixed to a molar ratio of 4:1:1:2 in a small test tube (hereafter 4112) and the solvent evaporated under nitrogen. For pH 7.0 the dry lipids were solubilized in a solution of 120 mM KH$_2$PO$_4$, 120 mM K$_2$HPO$_4$, 150 mM NaCl and 20 mM KCl. The solution was titrated to pH 7 with KOH. The molarity of total potassium is approximately 215 mM after mixing (1:1) with M2 vesicles containing pure water. This corresponds to a potassium activity inside the fused vesicles of 140 mM. It is lower than total potassium because of binding to phosphate. For pH 5.4, the dry lipids were solubilized in a solution of 120 mM K$_3$citrate, 120 mM KH$_2$citrate, and 120 mM NaCl titrated to pH 5.4 with KOH. The molarity of total potassium is approximately 300 mM after dilution. This correlates with a potassium activity of approximately 200 mM. Since the vesicles were always diluted 1:100 in translocation buffer during the assay, the Nernst potential for potassium after addition of valinomycin across the vesicular membrane will be the same at pH 7.0 and 5.4.

The suspension was mixed by vortexing vigorously for 10 minutes. It was then sonicated in a bath sonicator (Sonicor SC-4U, Sonicor Instrument Corporation, Copiague, NY) for 4 minutes and 30 seconds.
Preparation of M2 Mixed Lipid Vesicles

Equal volumes of 4112 vesicles and the M2 proteoliposomes were mixed (M2 mixed vesicles) at room temperature, vortexed briefly, and sonicated for 30 seconds. The combined solution was then frozen to -20°C, thawed at room temperature, and sonicated for 30 seconds. The freeze-thaw-sonication process was then repeated for a total of three cycles. The protein:lipid ratio was 1:10 in the new mixed vesicles with an internal buffer concentration reduced to fifty percent of the original concentration. Prior to experimental use the vesicles were sonicated and vesicle diameter measured by DLS.

Proton Flux Assay

Proton flux across the vesicle membrane was measured by the method described by Cao, et al. (33) and Franklin, et al (34). The translocation buffer for experiments at pH 7 was composed of 190 mM Na$_2$SO$_4$, 0.1 mM KH$_2$PO$_4$ and 0.1 mM K$_2$HPO$_4$ and, for experiments at pH 5.4, of 190 mM Na$_2$SO$_4$, 0.1 mM K$_3$citrate and 0.1 mM KH$_2$citrate.

Three milliliters of the translocation buffer were placed in the experimental cuvette and stirred to equilibrate the buffer at room temperature. A highly selective pH probe (AccupHast combination electrode model 13-620-297, Fisher Scientific, Hampton, NH) was inserted. A solution containing 30 µL of M2 mixed vesicles was next added to the cuvette and allowed to equilibrate for approximately 5 minutes at room temperature (~23°C) and changes in pH over time were recorded. After the baseline was stable for 2 - 3 minutes, 3 µL of the K$^+$ ionophore valinomycin (25 µg/ml ethyl alcohol, Sigma, Saint Louis, MO) were added to the solution. After 3 - 5
minutes, 7.5 µL of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP, 200uM in ethyl alcohol, Sigma, Saint Louis, MO), were added (Figure 2-2). Finally the solution was back-titrated after 3 - 5 minutes with 30 µL of 1 mM HCL. During the whole process the solution was constantly stirred while continuous pH readings were recorded. The concentration of valinomycin is not rate-limiting as changing its concentration did not significantly change the results. Inhibitor studies were performed in the presence of amantadine by adding 30 µL of 100 mM amantadine to a final concentration of 1 mM and then incubating the M2 mixed vesicles for five minutes prior to triggering proton translocation. The same procedure was followed to detect the change in the proton flux, namely addition of valinomycin followed by CCCP, and the standard back-titration.

To ensure the stability and integrity of the liposomes, control experiments with valinomycin added after 5 minutes, 15 minutes, and 45 minutes of the liposomes additions were conducted. Control liposomes were prepared in parallel without M2. Average vesicle diameter ranged from 173-218 nm, independent of protein content. Each M2 proton flux was measured with a control experiment under similar conditions.

The analog output of the pH meter was filtered at 20 Hz and amplified 200 times (model LPF-8, Warner Instrument Corp, Hamden, CT). The data were collected and stored at 100 Hz using LabVIEW software (version 7.0, National Instruments Corp, Austin, TX). In Excel the data were then average at 4 Hz. The data had a relative accuracy of 0.0002 pH units with a time resolution of less than one second. All tracings are scaled to the back-titration of 30 nmoles HCl done after each
experiment. Drift was subtracted out of each tracing to make the change in slope after valinomycin addition clearer.

*Calculation of Single Channel Proton Flux*

The time-average proton flux of a single channel was calculated from the initial rate of hydrogen influx, converted to current, and normalized to reflect both the extravesicular buffer strength using the back titration and the predicted number of functional M2 tetramers assuming full incorporation and tetramerization:

\[
i = \frac{J_{0,\text{norm}}}{N(\text{Tetramers})}
\]  

(1)

The initial rate of hydrogen influx was measured experimentally from the initial slope of the pH curve after valinomycin addition and was taken as an average of the steepest and the shallowest lines that could reasonably approximate the slope, which was then converted to hydrogen influx (moles/sec) based on the standard back-titration. A typical trace is shown in Figure 2-3. Based upon the total lipid and protein weight, the average surface area of a lipid molecule and the surface area of lipid vesicle, the number of M2 channels per vesicles can be calculated (Table 2-1). Vesicles that contain no or inactive M2 make up the CCCP signal and do not contribute to the initial valinomycin pH rise. The flux can be converted to conductance if the hydrogen driving potential is known. The solution inside the vesicles contains 150 mM K\(^+\) and the outside contains about 0.2 mM K\(^+\) after the addition of 30 µL of the vesicle solution to 3 mL translocation buffer. After the addition of valinomycin, the vesicle membrane is estimated to be initially clamped at a potential of approximately -112 mV. The time-averaged single channel conductance
and the single channel permeability were calculated for the initial symmetrical proton concentration conditions from:

\[ \gamma = \frac{I}{V_m} \]  \hspace{1cm} (2)

\[ P = \frac{RT}{(Fz)^2[ion]} \gamma \]  \hspace{1cm} (3)

The standard deviations are reported for all measurements. These experimental standard deviations are perpetuated, in the form of variances, to calculations of current and permeability using the following equation:

\[ \text{var} F(x, y) = \left( \frac{\partial F(x, y)}{\partial x} \right)^2 \text{var}(x) + \left( \frac{\partial F(x, y)}{\partial y} \right)^2 \text{var}(y) \]  \hspace{1cm} (4)

There were five protein-free controls pH 5.4 and four and pH 7.0. There were three M2 experiments at both pHs. There were two amantadine experiments at pH 5.4 and three at pH 7.0.

**Numerical Simulation of Proton Uptake**

Changes in external pH, internal pH, and membrane potential were simulated by numerical integration using the approach given in the Appendix. This traditional compartmental analysis represented the systems as two compartments, interior and exterior, separated by a membrane permeable to H\(^+\) and K\(^+\). The membrane potential is established from the equivalent circuit equation with selective permeability represented as selective conductance. Buffers were assumed to be in instantaneous equilibrium throughout both compartments and membrane permeability was assumed to be low enough relative to bulk diffusion to prevent concentration gradients in compartmental bulk solutions. Unless specified otherwise, the parameters used in the simulations were (aggregate) \(G_H = 0.000146 \text{ S (t = 0-5 min)}\) and \(G_H = 0.00146 \text{ S (t =} \)
5-10 min, Figure 2-7a-c only), $G_K = 0.00146 \ S (t = 0-10 \ min), [K^+]_i = 140 \ mM, [K^+]_o = 1.285 \ mM, pH_i = pH_o = 6.8, \ [Buffer]_i = 120 \ mM, [Buffer]_o = 1.188 \ mM, \ \text{trapped volume} = 2.2 \ \mu l$.

**Results**

*M2 induced pH change*

Our objective was to determine the proton permeation and amantadine sensitivity of the M2 protein at pH 7 and pH 5 in lipid vesicles. Previous researchers have induced proton flux into vesicles via an electrochemical gradient (1,34). The proton flux at such low concentrations of hydrogen is very minute. Using an assay similar to Franklin et al. (34), we directly measured the basification of a weakly buffered external solution when proton flux through M2 (reconstituted in lipid vesicles loaded with potassium) was induced by the addition of the potassium ionophore valinomycin.

Figure 2-2 shows the mechanism of proton transport across vesicles. Potassium efflux creates a potential of -112 mV inside the vesicles. This potential drives proton influx into vesicles with functional M2. The proton influx is recorded by measuring the increase in pH of the solution outside the vesicles. Proton influx was not detected in protein-free control vesicles. We did observe a small pH drift in some experiments, presumably due to buffer pK shifts or CO$_2$ solubility related to thermal equilibration, which has been subtracted from all tracings presented for clarity. Addition of the protonophore CCCP serves as a positive control for the presence of vesicles without active protein.
There is a clear increase in M2-mediated proton uptake as measured directly under neutral pH conditions using this method. Figure 2-3 shows a typical pH 7 result of pH change owing to proton translocation by M2. The fast rise in pH immediately after valinomycin addition in the vesicles containing M2 is due to proton movement in response to valinomycin induced K\(^+\) efflux. This fast signal is not observed in control vesicles. The 10 second slope after initial addition of valinomycin is proportional to H\(^+\) influx. This figure also shows the pH rise after CCCP addition with both M2 and control vesicles. This is due to the exchange of K\(^+\) for H\(^+\) in all vesicles that do not contain M2. The total signal is a combination of the valinomycin and CCCP signal which is proportional to the total volume entrapped inside the vesicles. Figure 2-3 also shows the effect of external amantadine on M2-induced proton flux. Consistent with amantadine block, the valinomycin signal is reduced indicating the reduction of M2-induced proton flux. Control vesicles treated with 1 mM external amantadine showed no amantadine induced leak at pH 7 (data not shown).

Experiments at low pH also verify the fact that M2 does not conduct significant numbers of Na\(^+\) or K\(^+\) ions but does conduct H\(^+\). Figure 2-4 shows the proton flux measurements at pH 5.4 using a citrate buffer. There is a detectable rise (proton flux) after addition of valinomycin in the M2 samples. The CCCP signal is similar to that seen at pH 7. The results with external amantadine show that the inhibitory strength of amantadine is decreased at low pH, as the difference in proton flux with amantadine and without amantadine is not very high.
Analysis of Vesicle Proton Flux

Proton flux through the M2 ion channel was calculated from changes in external pH on the basis of the rate of change in the external free hydrogen \([H^+]\) (calibrated as deduced from the back titration and the nominal number of tetramers in the experiment (Table 2-1). The average sizes of control vesicles, determined by dynamic light scattering, are not significantly different from those of M2-containing vesicles. The buffer capacities were similar for the two pH conditions, with the initial pH being 0.2 pH units below the buffer pK in each case.

Assuming a surface area of 63 A\(^2\)/phospholipid headgroup and the average vesicle diameters listed in Table 2-1, there were 5 - 8 x 10\(^{11}\) vesicles in the experimental samples, corresponding to a trapped volume of 2.2 - 2.8 µl. The amount of total protein used at pH 5.4 was 40.5 µg, while at pH 7.0 the total protein was 15 µg. Assuming that all of the protein was incorporated and in the tetramer configuration (functional), there were 1050 channels per average-sized vesicle in the pH 5.4 experiments and 250 in the pH 7.0 experiments. Table 2-1 shows that changing the pH from 5.4 to 7.0 has no significant effect in number of protons/tetramer/second.

A high concentration of amantadine (1 mM) was used to maximally quench M2 activity. Figure 2-3 shows that amantadine reduced the initial proton translocation rate, as quantified in Table 2-1. We see a 49±38 % reduction of M2 proton flux in the presence of amantadine at pH 7 (0.50 vs. 0.26 protons/tetramer-s). At pH 5.4 the amantadine sensitivity is reduced by 12 ±93% (0.24 vs. 0.21 protons/tetramer-s). Amantadine is known to block exclusively from the N-terminal side (4). Presuming
the orientation of the protein to be random in lipid vesicles, we only expect fifty percent orientated to each side and therefore a fifty percent block of proton conductance.

To calculate the single channel conductance, we divided the difference between the total proton influx per vesicle (determined from the back-titration-normalized external pH initial slope and the number of vesicles in the experiment) and that in the control experiment (due to a small amount of proton leakage through the bilayer), by the nominal number of channels in a single vesicle. At pH 7, the total proton influx into M2 containing vesicles after the addition of valinomycin was $124 \pm 63$ H$^+$/vesicle/second. Adjusting for number of channels (tetramers) and the membrane potential, this total influx gives a hydrogen flux of $0.50 \pm 0.29$ protons/tetramer-sec corresponds to a time-averaged single channel conductance of $0.72 \pm 0.42$ aS. At pH 5.4 these values are $257 \pm 122$ H$^+$/vesicle/second, $0.24 \pm 0.14$ H$^+$/tetramer/sec, and $0.35 \pm 0.20$ aS. The parameters for lipid vesicles are shown in Table 2-1. The proton permeability of M2 at pH 7 and 5.4 are $191 \pm 111 \times 10^{17}$ cm$^3$/s and $2.3 \pm 1.3 \times 10^{17}$ cm$^3$/s, respectively. These values are approximately 1000 times smaller than proton conductance by gramicidin. As calculated below, M2 has an open probability, $P_O$, on the order of 0.001. The gating of M2 may account for the low permeability determined here.

**Selectivity of M2**

The rise in pH after addition of valinomycin (the valinomycin signal) is inherently dependant on the maintenance of a potassium gradient. If the lipid membrane or M2 channels were to leak potassium, it would confound our studies. To
examine the leak of potassium through M2, we stirred the vesicles for 5, 15, or 45 minutes prior to the addition of valinomycin (Figure 2-5). The five minute pre-valinomycin stir time is standard for all experiments, allowing the reaction conditions to stabilize. The 15 minute stir shows a CCCP signal that is 13% reduced when compared to the 5 minute stir. Consistent with this observation, the 45 minute stirs show a 34% reduced CCCP signal. Protein-free control vesicles showed that even after 45 minutes the vesicles were stable, demonstrating that the lipid bilayers were tight enough to maintain the $K^+$ gradients. In contrast, introduction of valinomycin elicited an immediate pH increase. Clearly, valinomycin enabled the $K^+$ efflux necessary to drive proton flux through M2.

As a test of the degree of selectivity implied by the existence of a valinomycin signal, experiments were also performed with gramicidin, a known $H^+$-, $Na^+$-, and $K^+$-permeable channel. At a low gramicidin surface density (0.5 µg/mL), gramicidin exhibited proton flux into vesicles (Figure 2-6), presumably because the $H^+$ permeability is relatively high and the $K^+$ gradient was retained on the 5 min time scale. At higher densities, gramicidin eliminated the CCCP signal, presumably by leaking potassium from the vesicles. Of additional interest is the fact that, while using the same method of preparation, namely a 3X-freeze-thaw-sonication fusion of channel-containing and channel-free vesicles, the product shows a homogenous distribution of the channel, demonstrating vesicle fusion is essentially quantitative. At high concentrations of gramicidin, the CCCP signal is greatly reduced, indicating that few gramicidin-free vesicles persist after the fusion process.


Discussion

*M2-facilitated pH Change*

Vesicle uptake assays are inherently complicated because of the interactions of multiple driving forces. In the assay used here, we start with no pH gradient and with outward K\(^+\) and inward Na\(^+\) gradients. We then add a K\(^+\) ionophore to initiate the K\(^+\) efflux, which in turn leads to a negative membrane potential. The membrane potential drives proton influx into vesicles with M2 present, but not into tight, protein-free vesicles, which exhibit proton influx only after addition of CCCP (a protonophore). Using similar techniques with both valinomycin and monensin for K\(^+\) or Na\(^+\) gradients, respectively, Lin and Schroeder (1) used intravesicular pyridine fluorescence to demonstrate that vesicle pH modification (acidification or basification) proceeds as expected under the assumption that M2 is impermeable to Na\(^+\), K\(^+\), or other bath ions. We have measured the proton influx directly as a decrease in extravesicular [H\(^+\)] using a proton-sensitive electrode.

To help interpret the time course of pH changes, we simulated the flux through an ensemble of vesicles with total trapped volume, V, each containing H\(^+\) and K\(^+\) conductance pathways, using numerical integration to solve a system of buffer, equivalent circuit, and flux equations (see Appendix). The equations describe the relaxation of a system perturbed away from Donnan equilibrium at \(t = 0\). Because there are two permeable ions, the system returns to Donnan equilibrium, a state in which the Nernst potential for both ions equals the membrane potential, with a time course that depends on changes in ion content inside and outside the vesicles as a result of ion flux down electrochemical gradients. The equations neglect osmotic
effects, which are expected to be small because ion exchange is essentially obligate. This analysis demonstrates that one cannot determine the degree to which protons are more permeable than Na\(^+\) or K\(^+\) ions in such assays, and gives a clearer idea of the time course of the driving forces on the protons, so we present the results here to provide a context for our subsequent interpretations.

Our analysis assumes a set of conditions that are fairly typical and consistent with our experimental conditions. Namely, the solute concentrations were those used in our experiments, that the aggregate H\(^+\) conductance was 2\(\times\)10\(^{-4}\) S (similar to that observed in a typical experiment with 2\(\times\)10\(^{14}\) channels conducting 0.50 ± 0.29 protons per tetramer per second for an aggregate proton conductance of 1.46\(\times\)10\(^{-4}\) S), that pH = pK-0.2 inside and outside the vesicles, and that at time 0 the K\(^+\) conductance was increased by addition of valinomycin to 2\(\times\)10\(^{-3}\) S, with no other ions permeant. The calculated time courses of the change in free internal and external [H\(^+\)] are shown in Figure 2-7a. After increasing H\(^+\) conductance at t = 5 minutes, the external [H\(^+\)] drops slightly over the course of about one minute, whereas the internal [H\(^+\)] rises nearly 2 pH units over the same time course. We note that, in our experiments, even the high concentration of buffer inside the vesicles is still insufficient to hold the internal pH constant in the face of the large proton influx, even though buffering was assumed to be instantaneous. This is because, even with the high buffer concentrations used here, the K\(^+\) content of the vesicles, which contain only a small fraction of the total volume, exceeds the buffered H\(^+\). Nevertheless, the change in external pH is readily measurable.
Relaxation to the Donnan Equilibrium is attained after ~1 minutes. This is demonstrated (Figure 2-7b) by the time dependence of the Nernst potentials for $K^+$, $(E_K)$, $H^+$ $(E_{H^+})$, and the membrane potential $V_m$ under the same conditions as in Figure 2-7a, all of which merge at the equilibrium point. In Figure 2-7c, the time course of the external pH is amplified to show that the slow rise obtained due to the $G_H$ from M2 alone gives way to a rapid rise if additional $H^+$ transporters, such as CCCP, are added to the system.

Additional analysis with this simple mathematical model allowed us to determine the following properties of the experimental system:

1. The initial slope of the change of external $[H^+]$ vs. time is proportional to $G_H$, as long as $G_H < 10G_K$ (Figure 2-7d). After normalizing for buffer capacity (via the back-titration results), the time-average single channel current times the number of channels, i.e. $G_H$, is obtained directly from the initial slope as expected.

2. For greater values of $G_H$ (or lesser values of $G_K$), the initial rate of rise is additionally dependent on $G_K$, being proportional to $G_K$ when $G_K$ is rate limiting (data not shown). Also, the rate of rise in $pH_o$ varied dramatically if the initial $pH - pK$ is changed. Variations of the initial $pH_o$ from the buffer $pK$ are automatically compensated when one normalizes small external pH changes using a back-titration, but they could easily lead to factor of 5 or higher errors in the initial slope if not properly accounted for by the back titration.
3. The external pH reached a plateau when the membrane potential reached a constant value, corresponding to the establishment of Donnan equilibrium.

4. The height of the pH signal change depended strongly on the vesicular (trapped) volume (Figure 2-7e) and the inside and outside buffer concentrations (data not shown), but not discernibly on $G_{H}$.

5. Whether the equilibrium membrane potential is nearer to the initial $V_H$ or the initial $V_K$ depends primarily on the relative internal concentrations of buffered $H^+$ and $K^+$ (data not shown). Namely, it is closer to $V_H$ if there is more buffered $H^+$ inside and closer to $V_K$ if there is more $K^+$ inside. In the conditions used in our experiments, $V_m \approx -100$ mV (Figure 2-7b).

One might wonder what happens if the membrane is permeable both to $H^+$ and $K^+$ at the outset (i.e. if the M2 channel is imperfectly selective), say for $P_H > P_K$.

Although one might expect the driving force on protons to be small because the membrane potential approaches $V_H$, the electroneutrality-required obligate exchange still causes proton uptake under an outwardly directed $K^+$ gradient. If the internal free buffer content is less than the internal $K^+$ excess (relative to external $K^+$), a $[H^+]$ gradient will still develop and $V_H$ and $V_M$ will settle near to the original $V_K$ once the Donnan equilibrium is achieved. The kinetics of the relaxation in pH and $V_M$ are governed by the $K^+$ flux, and hence by the $P_K$ rather than the direct electrochemical driving force on $H^+$. This argument justifies the conclusions of Lin and Schroeder (1) concerning high M2 selectivity.

The argument does not apply directly to electrophysiological voltage clamp studies (e.g. Chizhmakov et al., (4)). To the extent that the cytoplasm pH and $[Na^+]$
are well buffered by the patch-clamp pipette, the cell will not relax to a Donnan equilibrium. However, it does raise some questions about the extent and timing of Na\(^+\) contamination of the very small volumes in the cytoplasm and patch pipette, which conceivably may become sufficient to produce an artifactually high apparent selectivity for H\(^+\) over Na\(^+\).

At the same time, the simulations help explain why gramicidin A, which is known to have a finite permeability to K\(^+\) and Na\(^+\) (around one-tenth that of H\(^+\)) can still yield a valinomycin signal on the several-minute time scale of our experiments. If permeability to H\(^+\) exceeds that to the metal ion, the initial driving force on H\(^+\) is low. However, if the internal K\(^+\) content exceeds the internal buffered H\(^+\), electroneutrality-required exchanges of H\(^+\) for K\(^+\) drive H\(^+\) into the vesicle until the internal buffer is overwhelmed, and a valinomycin signal is observable.

Quantitative Comparison to Previous Results

Lin and Schroeder (1) reported 7.3 protons per tetramer per second at pH 7.4 and a single channel conductance of 8 x 10\(^{-18}\) S (at 18 C), about 10X higher than our results. In our experiments, we took the initial slope from the 10 second interval starting 3 seconds after the addition of valinomycin. The initial jump (first 3 seconds) was ignored as an artifact of solvent addition rather than vesicle uptake. Lin and Schroeder do not mention any solvent artifact, but in the published figures there is a sharp discontinuity in slope during the first second and the second and subsequent seconds. The shape of the approach to equilibrium in our simulations (Figure 2-7c) is roughly that of an exponential, so it is clear that the relevant initial slope is that of the segment leading up to the Donnan plateau. Perhaps they focused on an earlier section
of the relaxation curve, and thus obtained a slope biased towards a higher value. This could explain why we estimate the time-average single channel conductance and permeability to be lower, \(7.2 \times 10^{-19}\) S at pH 7.0 at 22 C. Alternatively, our protein may be less active (incorporated or well-configured) than their’s. It is not possible from the current data to distinguish between these possibilities.

If one could make assumptions about the impact of pH on single channel conductance (via mass action) and acid-gating, it would now be possible to relate this to the measured single channel conductance of the open state, \(~6\) pS for this same preparation at pH 3 in planar bilayers (9). For instance, we could assume that the conductance of the open state is proportional to \([H^+]\) (with no saturation) and ignore any acid gating effects to extrapolate an open state conductance at pH 6.8 of 0.95 fS. By comparison to the measured time-average conductance of 0.72 aS, we would deduce that \(P_o = 7.7 \times 10^{-4}\), one order of magnitude larger than that observed in the planar bilayers (assuming that each experiment resulted from the fusion of one vesicle containing the nominal number of fully active tetramers), i.e. \(7.5 \times 10^{-5}\) (9). However, the acid-gating phenomenon has been well-established in electrophysiological experiments (4), saturation is known to occur below pH 8.5 (4,9), and the vesicle uptake results do not confirm the expectation of mass action, which point we discuss next.

\textit{Acid-activation}

From cell acidification (2), electrophysiological (4,30), and vesicle acidification (1) experiments, it is frequently stated that M2 is acid-gated. Solid state NMR measurements of the titration states of a peptide consisting of the
transmembrane domain from M2 (39) indicate that two of the four His residues in the selectivity filter are protonated with a pKa of 8.2, the third is protonated at pH 6.3, and the fourth is protonated at pH < 5. This indicates that it is the third His37 protonation that correlates with acid-gating in electrophysiological (4,7) and fluorescence (12,40) studies. Care has to be exercised in the interpretation of the effects of acidification on hydrogen conductance to distinguish the effects of mass action (passive electrodiffusion) from effects attributable to changes in protein conformation or dynamics. Nevertheless, a secure claim for acid-gating can be found in the result that basification of the extracellular fluid decreases outward H⁺ current through M2 channels in spite of an increased outward electrochemical driving force (2,4). The effect seems to be greatest when the N-terminus of M2 is exposed to the more basic solution, although some effect is seen in both directions (5).

In this regard, we were somewhat surprised that our measured H⁺ single channel permeabilities were not increased dramatically at lower pH. Lin and Schroeder (1) observed a 2-fold increase in flux at pH 5.7 (compared to pH 7.4) and we observed a slight decrease at pH 5.4 (compared to pH 6.8). However, we expect H⁺ influx to be increased at the lower pHs in both cases by a factor of 25 - 50 due to mass action, and by some additional factor because of acid gating. This lack of mass action and acid activation occurs in both studies, although they differ in protein species (Weybridge vs. Udorn), lipid membrane compositions, and palmitoylation and phosphorylation states of the protein (with Lin and Schroeder using the *Trichoplusia ni* insect cell expression in contrast to our usage of the *E. coli* bacterial expression system). Changes in pH over a about the same range typically lead to a ~10-fold
increase in proton conductance in electrophysiological studies with cell expression systems (e.g. (4)). Taken at face value, one could conclude that lipid protein interactions or conformational history causes different behavior in purified reconstituted systems and cell expression systems.

The observation that proton channels and transporters could have a constant $\text{H}^+$ flux over a large range of $\text{H}^+$ bulk concentrations has been noted in other systems. Many studies with proton transporters (41) suggest that proton transport is pH-independent in physiological conditions. For instance, Feniouk et al. (42) recently measured proton conductance for $\text{F}_0$ in chloroplasts, finding it to have a weak dependence on pH and a higher unitary conductance than expected from gramicidin measurements. They attributed these behaviors to proton buffering by protein side chains along the transport pathway. In this case residues near or in the selectivity filter may have a buffering role. Similar behavior is shown at near-neutral pH by proteins that form channels as summarized in DeCoursey (41). One might consider that the pH near the mouth of the channel is buffered by the lipid head group region or that the kinetics of proton approach to the channel are modulated by bulk buffer (43).

We suppose that in our experiments an obligatory site in the transport pathway is saturated by pH 7.0 whose $\text{H}^+$ dissociation rate constant is approximately the same at pH 5.4 as at 7.0. It is possible that acid-activation may occur above pH 7.0 or below 5.4.

*M2 Variants*

In addition to those data shown, we have conducted preliminary proton flux studies with various mutants of M2, including one with the His$_6$ tag used for
purification in either the N-terminal or C-terminal positions, without the His\textsubscript{6} tag (TEV-cleaved), and with fluorination of Trp41. All show similar signals indicating that these mutated samples also primarily conduct protons and that the proton flux is not affected by these mutations. Proton flux studies with reconstituted Udorn M2 provided by Dr. Larry Pinto’s group and reconstituted Weybridge M2 provided by Dr. Alan Hay’s group have also been studied in our lab with similar flux results. From these results, it appears that the quality of the incorporation and the level of functionality are similar for all three preparations.

*Amantadine Block*

Preincubation of the sample with 1 mM extravesicular amantadine resulted in a lower initial slope after addition of valinomycin, which corresponds to a final flux of 0.26 ± 0.12 protons per tetramer per second at pH 7.0 and 0.21 ± 0.19 protons per tetramer per second at pH 5.4. These equate to a single channel conductance of 0.37 ± 0.17 aS and 0.31 ± 0.28 aS, respectively. Thus, M2 is inhibited 49 ± 38% at pH 7.0 and 12 ± 93% at pH 5.4. Although the M2 affinity for amantadine is known to be 10 \( \mu \text{M} \) (30) we chose to use 1 mM to obtain maximal block. At such high concentrations of amantadine, we expected 100% block of M2 for those channels with the N-terminus of the monomers projecting out of the vesicles (4). Lin and Schroeder established that M2 in their preparations was randomly oriented such that half of the N-termini were inside and half outside. Assuming that the M2 tetramers in our proteoliposomes are also randomly oriented, we expect proton flow to be inhibited by 50% as we observed. The degree of block was reduced at pH 5, as expected from electrophysiological experiments where \( K_i \) increases ~50% upon change in pH from
7.5 to 6.2 for Udorn M2 (30). This reduction is not due to titration of amantadine, which has a $pK_a$ of 10.1 and should be fully protonated at both pH 5.4 and 7.0.

Selectivity of M2

Lin and Schroeder estimated, based on the lack of baseline drift and contrary ionophore signal, that M2 was essentially perfectly selective for protons in the presence of high concentrations of potassium. We evaluated this conclusion in our system by stirring the proteoliposomes for 5, 15, or 45 minutes prior to addition of valinomycin (Figure 2-5). The 15 minute stir showed a 13% reduction in total signal compared to the 5 minute stir and provides evidence that, on the relevant time scale (10 minutes), the vesicles remain relatively selective against potassium.

A 34% reduction in total signal after stirring for 45 minutes compared to 5 minutes was also observed at pH 7. Protein-free liposomes did not show reduced signal even after a 45 minute pre-valinomycin stir (data not shown). This suggests that, in addition to protons, M2 also transports other ions, including potassium. Assuming that all vesicles in the experiment represented in Figure 2-5 lost 34% of their membrane potential driving force, due to a reduction in $V_K$, over 40 minutes, we estimate that the $K^+$ influx over the 40 minute period was 0.25 ions/tetramer/second. The $K^+$ flux is equivalent to a permeability of $8 \times 10^{-23}$ cm$^3$/second. Comparing permeabilities, M2 is selective for $H^+$ over $K^+$ by a factor of $\sim 10^7$.

To examine the dependence of our assay on the selectivity of M2, experiments were also performed with gramicidin A (Figure 2-6), which is selective for $H^+$ over $K^+$ by a factor of $\sim 10$ (44). In our experiments, when gramicidin was reconstituted into liposomes at concentrations of 50 - 500 ng/mL, the entire potassium gradient
(membrane potential) was lost after only 5 minute incubation and stirring in the low 
K⁺ buffer. At the lower dose of gramicidin (5 ng/mL) the proton influx is similar to 
that observed with M2, indicating that even with a moderate amount of nonselective 
leakage, preservation of a significant portion of the potassium gradient over 10 
minutes is possible.

Incorporation, Tetramerization, and Open State Probability

We do not yet have a good measure of incorporation and tetramerization for 
M2 reconstituted into vesicles, but preliminary evidence from NMR studies indicate 
that protein incorporation is variable and incomplete under conditions used to date. 
However, to provide upper limits, we will continue the assumption of complete 
incorporation used previously, extending it as well to gramicidin A incorporation, and 
examine how the slope in the M2 signal compares to that of the gramicidin A signal.

At 5 ng gramicidin A/mL there are approximately 6 gramicidin monomers per 
vesicle or 8 x 10⁻¹¹ mol cm⁻². Assuming the dimerization constant determined with 
dansylated gramicidin fluorescence studies (K = 2 x 10¹³ mol⁻¹ cm² in painted 
dioleoylphosphatidylcholine bilayers, (45), at this concentration the equilibrium is 
heavily biased (99%) towards dimers, yielding 3 dimers per vesicle. For M2, the 
tetramer→dimer dissociation constant was measured with analytical centrifugation 
(46) to be 4x10⁻²¹ M. To determine the fraction of tetramers in our experiments based 
on the Kochendoerfer et al. model (46), we compared the lipid concentration (15 mM) 
and the M2 monomer (MW 11,250) concentration (e.g. 0.09 mM at pH 7), to those of 
the concentrations of detergent (15 mM dodecylphospho-choline) and M2 protein 
(covering a range) in the Kochendoerfer et al. (46) micelle preparation. According to
their model analysis, the tetramer would comprise a protein weight fraction of ~0.9, with a fraction of ~0.1 containing monomers and octamers at our protein density. Hence, for a typical vesicle at pH 6.8, 90% of the mass or 222 tetramers per vesicle would be in the tetramer state. Qualitatively, it appears that the initial H\(^+\) flux with 5 ng/ml gramicidin A, corresponding to 3 channels/vesicle (Figure 2-6), is similar to that with M2, suggesting that gramicidin A is 74x more active than M2. The single channel conductances for the two channels are similar at lower pH (9), so we attribute the higher activity of gramicidin to a higher probability that the dimer channel is open, i.e. in the conducting state. On this basis, if for the conditions of peptide density used here, the Po for the gramicidin dimer is 1.0, we estimate the Po for the M2 tetramer to be 0.014, similar to values estimated from single channel conductance studies (9).

In summary, the proton flux assay has the potential to provide very sensitive and accurate measurements of M2 channel activity. The present work reports our first attempts to measure proton flux through the M2 protein reconstituted into lipid vesicles. There are many parameters that are yet to be studied, such as dose response curves for M2 and amantadine, internalization of amantadine, reconstituting M2 by various methods such as dialysis, more thoroughly measuring proton flux with changes in pH for studying acid activation and His37 titration, and evaluation of M2 protein orientation in smaller vesicles. Future research will lead to finer control of these variables.
Appendix

We assumed that buffer equilibration was instantaneous on the time scale of membrane flux. The analytical solution to the differential equation was assumed to be too difficult to obtain due to the contributions of driving forces for two ions, one being buffered according to:

\[ HP \leftrightarrow H^+ + P^- \]

\[ K_d = \frac{[H^+][P^-]}{[HP]} \]  

(A1)

The algorithm used was:

A. From the initial pH, inside and outside of the vesicles, the initial total \([H] = [H^+] + [HP]\) (i.e. free hydrogen plus hydrogen buffered by phosphate) is first calculated from the free \(H^+, [H^+],\) and the total buffer (phosphate) concentrations, \([P],\) on each side. For this, Equation A1 is converted to a quadratic equation in \([H^+]\) with only \([P] and [H]\) as parameters by substituting \([H^+]-[H]+[P]\) for \([P]\) (the unprotonated buffer) and \([H]-[H^+]\) for \([HP]\), both based on conservation of matter, into equation A1 to obtain:

\[ [H^+]^2 + ([P] - [H] + K_d[H^+]) - K_d[H] = 0 \]  

(A2)

Solving (A2) for \([H],\)

\[ [H] = \frac{K_d + [H^+] + [P]}{1 + K_d[H^+]} \]  

(A3)

Because the first two terms of the numerator are negligible, the bound and free proton concentrations comprise approximately one half the total buffer concentration
when pH=pK, as expected. This equation allows us to explore conditions where pH≠pK.

B. The initial membrane potential is computed for selected conductance parameters using the equivalent circuit equation, with the assumption that only H⁺ and K⁺ are permeant, and computing the Nernst potentials, $E_H$ and $E_K$, from the initial concentrations inside and outside of the vesicles:

$$V_m = \frac{G_H E_H + G_K E_K}{G_H + G_K}$$  \hspace{1cm} (A4)

C. The flux for each species, in moles per second, is taken from the ionic current using the same conductance and driving force parameters:

$$J = \frac{G_S}{F} (V_m - E_s), \quad S = H^+ \text{ or } K^+$$  \hspace{1cm} (A5)

D. The change in total concentration for each ion on each side in a short time, $\Delta t$, is taken as

$$\Delta[S] = \frac{J \Delta t}{V}, \quad S = H^+ \text{ or } K^+$$  \hspace{1cm} (A6)

where the sign of the change depends on the direction of flux, and $V$ is the volume of the compartment being calculated, intra- or extra-vesicular. The time step must be small enough to allow only an incremental change in ion concentrations on each side.

E. Finally, the new free H⁺ concentration is computed in each compartment, assuming instantaneous buffer equilibration, from the new [H] inside and outside using the rational solution of equation A2, namely:

$$[H^+] = (-A + (A^2 + 4K_H[H])^{\frac{1}{2}}) / 2$$  \hspace{1cm} (A7)
where \( A = [P] - [H] + K_p \)

This algorithm was then iterated repeatedly until a steady state was achieved. The initial point was taken as the time of addition of valinomycin to create \( G_K > 0 \), assuming a preexisting \( G_H > 0 \) via M2 channels.

**Acknowledgements**

We acknowledge the help of Brad L. Rogers, Lane D. Squires, and Steven D. Later with experiments and simulations and funding from NIH grants AI23007 and GM61272.
Reference List


**Figure 2-1** – SDS-PAGE of Reconstituted M2. Lane 1 - the apparent molecular weights (kDa) of a standard ladder. Lane 2 - M2 reconstituted in vesicles (DMPC:DMPG 4:1) is free of contaminants. Lane 3 - Application of trypsin to proteoliposomes cleaves M2.
Figure 2-2 – Proton Flux Assay. Proton flux is driven by a membrane potential created when valinomycin is added to vesicles prepared with asymmetric [K⁺]. Valinomycin, a potassium ionophore, allows potassium efflux, creating the potential that drives proton influx through M2 protein. Proton-leaky vesicles without M2 also will exhibit flux at this step. CCCP, a protonophore, permits proton influx into vesicles that did not previously discharge their gradient. The pH stabilizes after addition of valinomycin when vesicles reach the Donnan equilibrium for hydrogen and potassium. Activity of K⁺ was determined as described in methods.
Figure 2-3 – M2 Proton Flux at pH 7.0. Representative tracings of proton flux into vesicles: A) Protein-free vesicles show no change in slope after addition of valinomycin confirming that these vesicles do not leak protons. Addition of valinomycin is indicated by the arrow and addition of CCCP is marked by the arrowhead. The presence of a CCCP signal shows the influx of protons into control vesicles. B) Vesicles containing M2 show increased proton influx after addition of valinomycin. C) This proton influx can be reduced by pre-incubating M2 vesicles with amantadine.
Figure 2-4 – M2 Proton Flux at pH 5.4. Representative tracings of proton flux into vesicles: A) Protein-free control vesicles show no change in slope after addition of valinomycin. A second addition of valinomycin seemed to cause a little artifact in control vesicles. B) M2 increased proton influx after addition of valinomycin. C) Amantadine block is less effective at pH 5.4. The meaning of arrows and arrowheads is the same as in Figure 2-3.
Figure 2-5 – Time Series. Vesicles were stirred for 5, 15, or 45 minutes prior to the addition of valinomycin. A) 5 minute pre-valinomycin stir is our normal protocol. B) 15 minute pre-valinomycin stir showed a 13% reduction in total signal compared to 5 minute stir. C) 45 minute stir show a 34% reduction in the total signal compared to 5 minute stir. As the control vesicles showed no reduction even after 45 minutes, the reduction seen in proteoliposomes is attributed to leak of potassium through M2. The meaning of arrow and arrowhead is the same as in Figure 2-3.
Figure 2-6 – Effect of Gramicidin on the proton flux assay. Vesicles containing gramicidin were prepared in a manner similar to M2 containing vesicles. The final concentration of gramicidin was 0, 5, 50, or 500 (not shown) ng/mL. A) Protein-free control shows no valinomycin signal. B) 5 ng/mL of gramicidin showed twice the slope after addition of valinomycin as did control. It also shows a reduced total signal, suggesting potassium leakage through gramicidin. C) 50 ng/mL of gramicidin was sufficient to eliminate any response to valinomycin and CCCP. Higher concentrations also showed no signal (not shown). The meaning of arrow and arrowhead is the same as in Figure 2-3.
Figure 2-7 – Theoretical prediction for the rate of establishment of Donnan equilibrium in a system with two permeable ions. At time zero, $G_K$ is increased to represent the addition of valinomycin; with $G_K=0$, baseline is flat prior to this point. The increase in $G_H$ at the midpoint is intended to represent the addition of CCCP to stimulate the relaxation to equilibrium. a) The external (bath) pH rises slightly, while the internal (intravesicular) pH falls rapidly, particularly after the increase in $G_H$ at t=5 min. b) Acidification of the vesicles is accompanied by a loss of driving force on $H^+$, as shown by the decline in the Nernst potential for $H^+$, $V_H$. The membrane potential, $V_M$, is sandwiched between $V_H$ and the Nernst potential for $K^+$, $V_K$. At t=5 min, $V_M$ abandons its proximity to $V_K$ temporarily because $G_H$ is set to $G_K$ so $V_H$ contributes more heavily to the equivalent circuit equation for $V_M$ (equation A4).
c) Expanded view of the external pH trace on a scale similar to that used for experimental traces. The 10x increase in $G_\text{H}$ at 5 minutes is intended to represent CCCP addition. Differences in shape between calculated and experimental traces could reflect membrane incorporation times for valinomycin or CCCP, which are assumed to be instantaneous in the simulation.
d) Increased $G_H$ results in complete Donnan equilibration in <5 minutes. The initial slope is proportional to $G_H$. e) The height of the valinomycin peak depends on the trapped volume, as shown here for volumes given in µl, as well as on internal non-protonated buffer concentration and internal $[K^+]$ (data not shown). The initial slope is not dependent on these factors, as long as it is determined during the first 20 s after initial ionophore addition. For this simulation, $G_H$ was set to 0.0006 S (with $G_K$ being 10x higher) to speed equilibration.
<table>
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<td>H⁺/Tetramer-s</td>
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<td>Proton Permeability (cm²/s x10¹⁷)</td>
<td>2.3 ± 1.3</td>
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**Table 2-1** – Calculation of Single Channel Current, Conductance, and Permeability.

*a* Assuming 63 Å² per headgroup per leaflet x 2 leaflets per bilayer.
Chapter 3 – Determination of the Stoichiometry of DCCD Block of Proton Flux through $F_O$ Following Deletion of an *E. coli* Gene Suspected of Forming a Confounding Cation Channel

Abstract

The proton-translocating $F_OF_1$ ATPase (ATP synthase) is a member of the ATPase protein family that bioenergetically interconvert proton motive force with the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (Pi). $F_O$ is the membrane sector that translocates protons ($H^+$) across membranes, converting electrochemical energy to rotational inertia, thus driving ATP formation in $F_1$. Previous experiments have been partially confounded by a contaminating channel, $C_L$, which co-purifies with $F_O$ and leaks cations. In this chapter $C_L$ activity is shown to not decrease following deletion of the previously uncharacterized *yraM* open reading frame of *E. coli*. In fact, deletion of this reading frame actually increased apparent $C_L$ activity. $F_O$ purified from a strain in which *yraM* was deleted is just as active as $F_O$ purified from the wild-type strain. $F_O$ from the deletion strain was utilized to examine the single-hit hypothesis of DCCD inhibition of passive proton flux through $F_O$. A DCCD-induced reduction in ATP synthase activity correlates with a reduction in the total initial slope, the number of functional $F_O$ per µg protein, and the single channel proton flux. The data suggest that passive proton flux requires the addition of 2 DCCD per $F_O$, to totally inactivate passive proton flux, while ATP synthase activity requires 1 DCCD per $F_O$ for inactivation.
**Introduction**

The FO\(_{1}\) ATPase (ATP synthase) is a member of the proton-translocating ATPase protein family that bioenergetically interconverts proton motive force (PMF) with the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (Pi). ATP synthase is vital for life in organisms from both the plant and animal kingdoms and different members of this family of enzymes can be found in bacterial cytoplasmic membranes, mitochondrial inner membranes, and thylakoid membranes of chloroplasts, and archaeabacteria. ATP synthase catalyzes the last step in oxidative metabolic pathways using a proton electrochemical gradient to make ATP from ADP and phosphate, as postulated forty years ago by P. Mitchell. It is noteworthy that this mechanism can run in the reverse direction so that ATP hydrolysis can be used to pump protons up an electrochemical gradient within cells (F-type ATPase in bacteria) or organelles (V-type ATPase in eukaryote cells). The eukaryote F-type ATP synthase has not been shown to run in the reverse direction.

The structure of FO\(_{1}\) ATPase is described in Chapter 1. The FO\(_{1}\) sector is the site of ATP formation or hydrolysis. Its structure and function are well known (1,2). It is composed of three sets of \(\alpha\) and \(\beta\) subunit pairs and one each of \(\gamma\), \(\delta\), and \(\varepsilon\). A conformational shift in \(\gamma\) subunit alters the interactions of the \(\alpha\beta\) pairs, causing ATP formation. \(\gamma\) and \(\varepsilon\) are linked to the c subunit ring of FO\(_{0}\). By labeling the \(\gamma\) subunit Noji et al (3) demonstrated that the \(\alpha\beta\) subunits rotate with respect to the \(\gamma\) subunit.

FO\(_{0}\) is the proton transport sector that is embedded in the membrane and connected to the FO\(_{1}\) sector which sits above the membrane. FO\(_{0}\) is composed of a ring
of c subunits, two b subunits, and one a subunit with a total molecular weight of 136 or 160 kDa if there are 9 or 12 c subunits respectively (molecular weights of a, b, and c are 30, 17, and 8 kDa, respectively (4)). The ring of c subunits spins in the plane of the membrane, carrying the protons through the membrane. This spinning is coupled to the F₁ sector through the γ subunit. Sambongi et al. have published the visualization of the spinning of F₀ coupled to F₁ (5). The a and b subunits are together on the outside the c subunit ring. The functional transport of protons by F₀ is sometimes compared to gramicidin (6). Although the mechanism of proton transport is different (discontinuous water wire versus a continuous one, respectively) the functional form of both channels are composed of two half channels. In the case of F₀ both halves of the channel are in the a subunit. Protonation and subsequent rotation of the protonated c subunit 360° around the ring forms the link between the two half channels. Based on available structural data, Aksimentiev et al. (7) recently modeled the interactions between the a and c subunits. The studies confirm that Asp61 of the c subunit is the residue that is protonated during proton translocation. Using the assumption that Ser206 in the a subunit is the termination of the cytoplasmic half channel and that Asn214, also in the a subunit, is the termination of the periplasmic half channel, it was shown by Aksimentiev et al. (7) that a hydrogen-bonded intermediate was formed when two protonated c subunits are close to Arg210 of the a subunit. The hydrogen bonding extends from Ser206 to one protonated Asp61 to Arg210 to a second protonated Asp61 to Asn214. When only one c subunit is protonated a strong ionic attraction occurs between Asp61 and Arg210 such that rotation is stopped. Only when the second Asp61 is protonated is the weaker
hydrogen bond formed, allowing for rotation of the c subunit past Arg210. The authors proposed that this bridge is a ratchet that hinders Fo from rotating in the improper direction and that the protonation of Asp61 in both c subunits is dependent on the concentration of protons on their respective side of the membrane. This mechanism explains passive proton diffusion and activity as an ATP synthase, but seems to run counter to the use of ATP to pump hydrogen up a gradient, therefore, it may be a better model for the eukaryote F-type ATP synthase but not the larger family of proton-transporting ATPases which can run in reverse.

The bacterial FoF1 ATPase is particularly interesting to study because it is a true motor in the classical sense. Like an electrical generator, ATP synthase has a rotor (c ring, γ and ε subunits) and a stator (α, β and δ subunits) (8). Rotation of c with respect to a and b is then transferred to the stalk (γ and ε) producing conformational changes in α and β to make ATP.

The energy that drives this molecular motor is the proton gradient that is created by mitochondria, chloroplasts, and bacteria. Several previous studies have calculated total proton flux through a known amount of purified Fo from E. coli (after F1 has been stripped off). Most of these have utilized Fo containing liposomes where the proton flux rate was measured while under a transmembrane potential (9,10,11,12,13,14). Although estimates vary by a factor of 1,000 (Cao 70 H+/Fo/sec, Lill 10^5 H+/Fo/sec), the two most recent publications vary only by a factor of 2 and estimate the flux to be 6240 protons per second per Fo (6) and 3100 protons per second per Fo (12). These calculations represent the time-averaged single channel
conductance of \( F_O \) after the \( F_1 \) sector is removed. In this chapter I report the single channel flux in a mutant strain of \( E. coli \).

In the earlier measurements of the single channel conductance of \( F_O \), Cao et al. (13) and Franklin et al. (12) were confounded by a contaminating protein that co-purified with \( F_O \) and leaked cations. Characterization of this leak-channel revealed a cation channel named \( C_L \). The channel was characterized by Cao et al. and found to hinder their assays since it leaks \( K^+ \), eliminating the membrane potential in some of the vesicles. To calculate the single channel conductance, our proton flux assay relies on using the Poisson distribution to calculate the number of active \( F_O \) channels per vesicle. Since the \( C_L \) channel reduces the total signal of the assay by reducing the total number of observed vesicles, less protein must be used during vesicle preparation to avoid adding so much \( C_L \) that the total signal is completely eliminated. This reduces the maximum proton flux, making it more difficult to detect proton flux from background noise. Their assay is very similar to our current protocol.

In an attempt to improve the sensitivity of our proton flux assay, the gene for \( C_L \) was potentially identified and deleted by Dr. William Brusilow and co-workers. The amino acid sequence of this purported channel matched the \( E. coli \) yraM open reading frame (GenBank accession number AAC76181) that prior to our studies had no known function, but was thought to be a lipoprotein, a glycosidase, or have another unknown function. The protein is expected to have a mass of 72-73 kDa. Quantification of proton flux through \( F_O \) purified from this new deletion strain and experimental confirmation of the deletion of \( C_L \) were the aims of this chapter.
This new strain was then used to assay the stoichiometry of inhibition of $F_O$ by $N,N'$-Dicyclohexylcarbodiimide (DCCD). As this drug has been shown to inhibit $F_O$ function (15), the ability of DCCD to block proton flux is used to confirm that the proton flux is due solely to $F_O$. Researchers have been examining the mechanism of inhibition for decades and have found that DCCD inhibits ATP synthesis by covalently binding to Asp61 on the c subunit to form an N-acyl urea (16). Asp61 is the residue that carries the proton through the c subunit ring. Thus DCCD selectively blocks proton transport by $F_O$. The stoichiometry of DCCD inhibition of $F_O$ required to block proton flux has been debated. There are two generally accepted models of inhibition.

The first model is the single hit hypothesis which suggests that no matter how many c-subunits constitute an active $F_O$ proton channel, it only requires one DCCD molecule per $F_O$ to entirely inhibit proton transport. Much research from the laboratory of R. Fillingame supports this hypothesis (17,18,19,20,21). In Hermolin and Fillingame (19) it was shown that 1 DCCD/$F_O$ is sufficient to inhibit ATPase activity. Using HPLC chromatography to separate the DCCD modified c subunits from the unmodified subunits and using specific radioactivity of c subunits treated with radioactive DCCD, they provide support for the single-hit model. Dmitriev et al. (17) incorporated non-functional c subunits into $F_O$ and observed how the activity of $F_O$ was affected. Using two forms of the c subunit and the assumption that the reassembled complex was either completely functional or completely nonfunctional, they found that it took either 1 or 2 c subunits to inactivate $F_O$. Jones et al. (21) showed that incorporation of one c subunit with a Asp61Gly mutation appears to
inhibit $F_O$ activity. However mutation of Asp61 to the structurally similar, but non-protonatable, Asn showed only reduced inhibition with incorporation of one c subunit. The mechanism of how DCCD binding to residue 61 of the c subunit inhibits proton flux through $F_O$ has never been clearly established, but Asp-61 is integral in the proton flux pathway. It is likely that DCCD binding at this site prevents proton binding.

A second possible model has some support in the literature, particularly from the laboratory of L. Ernster (22,23). It is possible that $F_1$, when attached to $F_O$, slows down proton flux thereby making it easier to inhibit proton flux with DCCD than inhibition of proton flux through $F_O$ alone. Kopecky et al. (23) also used specific radioactivity to show that while only 1 DCCD per $F_O F_1$ complex was sufficient to inhibit ATPase activity, 2 DCCDs per $F_O$ were required to inhibit $F_O$ when $F_1$ was absent. Similar results were reported by Glaser et al. (22), who created titration curves of proton translocation coupled to ATPase activity and NADH oxidase activity of the $F_O F_1$ complex and passive proton flux through $F_O$ alone to support their conclusions.

Two other possible results are not supported in the literature, yet are suggested by the stoichiometry of $F_O$. One is that DCCD simply inhibits proton flux by out competing hydrogen at aspartate-61. Consequently, addition of more DCCD should lead to more inhibition. The last possibility is that there is no inhibition of proton flux until all c subunits have been hit by DCCD. This last case is not likely as there are approximately 10 c subunits per $F_O$. Since all the published values suggest 1 or 2 DCCD per $F_O$, it is very unlikely that they are all off by a factor of 10.

In this chapter we examine the single-hit model of DCCD in a system that has never been utilized before. First $E. coli$ membranes containing the entire $F_O F_1$ complex...
complex are treated with DCCD. The ATP synthesis activity of the membranes is used to determine the block of $F_0$ by DCCD. Then $F_0$ is stripped from the complex and proton flux assayed. The quantitative proton flux assay developed in our lab (12) is ideally suited to test the single-hit model, particularly to examine whether one or two DCCD molecules are required to inhibit $F_0$ activity. The proton flux assay we perform is unique in that we can directly correlate the activity of individual $F_0$s with proton flux.

**Materials and Methods**

*Creation of a potential $C_L$ deletion *E. coli* strain*

The $C_L$ deletion mutant was a kind gift from William Brusilow, Ph.D. (Wayne State University School of Medicine, Detroit, MI). Briefly, the $C_L$ deletion mutant was constructed by following the Wanner deletion method (24). This method allows for insertion of an antibiotic resistance gene in a chromosomal gene. A double-stranded mutagenic oligonucleotide was transformed into the Wanner strain and allowed to recombine, replacing the antibiotic resistance, resulting in an otherwise wild type operon with the yraM gene deleted. The deletion strain was selected for by loss of ampicillin resistance and its identity confirmed by DNA sequencing. The constructs were then co-transfected into an *E. coli* ATPase deletion strain with $ilv::Tn10$ and PCR. PCR was used to screen for restoration of the unc operon.

*Isolation of $F_0$*

Two tubes of 10 mL each of autoclaved LB broth were inoculated with either *E. coli* containing our $C_L$ deletion or wild type *E. coli*. The tubes were then placed in
a shaking incubator at 37° C. The OD (optical density) of a 1:10 dilution of bacteria in water was measured at 600 nm hourly until the bacteria grew to a corrected OD of 5. The two tubes were then divided between four 2-liter Erlenmeyer flasks each containing 500 mL of autoclaved LB broth and the bacteria were again incubated at 37 °C with vigorous shaking. The OD was tested hourly until the bacteria grew to an OD of 0.8 and then the broth was tested every half hour. When the bacteria reached an OD of ~1.5, the cells were pelleted by centrifugation for 10 minutes at 10,000 x g. The supernatant was discarded and the pellets were removed and combined into one pre-weighed tube and resuspended in enough Buffer 1 (containing 50 mM Tris-Cl and 10 mM MgCl$_2$ pH 8.0) to yield 10 grams of pellet/15 mL buffer. The cells were then ruptured by extruding them through a French Press twice at 16,000 psi. The ruptured cells were then centrifuged for 10 minutes at 10,000 x g after which the pellet was discarded. The supernatant was centrifuged for 60 minutes at 100,000 x g. A small portion of the pellet was then put in MOPS-Mg solution (containing 50 mM Tris-Cl and 10 mM MgCl$_2$ pH 7) on which a Lowry Protein assay and ATPase assay were later performed. The Lowry was only performed so that the specific ATPase activity could be determined. This activity should be reduced after performing following process to strip F$_1$ off of the membrane bound F$_0$.

The remaining pellet was resuspended in 25 mL of Buffer 3 (containing 1 mM Tris-Cl, 0.5 mM EDTA, 10 % glycerol and 0.1 mM AEBSF pH 8.0). The solution was then centrifuged for 60 minutes at 100,000 x g. After centrifugation, the supernatant was discarded. A small measured portion of this pellet was also suspended in 100 µl of MOPS-Mg solution for protein and ATPase assays. The
remaining pellet was resuspended in 25 mL of Buffer 3 and centrifuged for 60 minutes at 100,000 x g.

After centrifugation the supernatant was discarded and the pellet was resuspended in 2 mL of buffer 7 (containing 50 mM Tris-Cl, 10% glycerol, 0.1 mM EDTA, 0.1 mM AEBSF, and 0.1 mM DTT pH 8.0). Deoxycholate was added to a concentration of 0.1% and the solution was stirred for 10 minutes on ice in the dark. The solution was then centrifuged for 60 minutes at 100,000 x g. The supernatant was discarded and the pellet was resuspended to the same volume as before. Deoxycholate was again added to a concentration of 0.1% and the solution was stirred for 10 minutes on ice in the dark. The solution was then centrifuged for 60 minutes at 100,000 x g. The supernatant was discarded and the pellet was resuspended in 2 mL of Buffer 7. A Lowry assay was then performed on this and the previously saved aliquots.

The final F0 solution was diluted until the protein concentration, as determined in the Lowry, was ~20 mg/mL. The volume of the solution was then measured and 0.13 mL of saturated (NH4)2SO4, 65 µl of 10% sodium cholate, and 95 µl of 20% octyl glucoside were added per mL of solution. This final solution was stirred on ice for 20 minutes.

The solution was the centrifuged for 60 minutes at 100,000 x g. The supernatant was saved and its volume measured. 0.538 mL of saturated (NH4)2SO4 was added per mL of measured supernatant. The solution was then stirred on ice for 20 minutes until a white precipitate formed. The solution was then centrifuged for 10 minutes at 12,000 x g. The supernatant was discarded and the pellet resuspended in
0.5 mL of Buffer K (containing 10 mM Tris-Cl, 150 mM NaCl, 10% glycerol, 0.2 mM AEBSF and 1% sodium cholate pH 8.0). A Lowry assay was performed to find out the final protein concentration and an SDS-PAGE gel was run to verify the protein purity. The final protein solution was stored in liquid N₂ and thawed just before use.

**Vesicle Preparation**

Vesicles were prepared from *E. coli* Polar Lipid Extract (Avanti Polar Lipids, Alabaster, AL catalog # 100600P) and 5% (wt:wt) cholesterol. The lipids were solubilized (30 mg/mL) in Dialysis Buffer (containing 150 mM KH₂PO₄ and ~100 mM KOH titrated to pH 7.00 and filtered at 0.1 µm) to which 2 mM β-mercaptoethanol and 1.5 % octyl glucoside were added. The reducing agent and detergent were added after filtering. The lipids were placed on a rocker at medium speed for 3 to 4 hours to complete dissolution. The lipid solution was dialyzed (12 – 14 kDa cut off limit, Spectrum Medical Industries, Inc., Laguna Hills, CA) against 1 L Dialysis Buffer. The lipids dialyzed for over 30 hours in a darkened cold room with the Dialysis Buffer being changed at least twice during that period. The resulting vesicle suspension was aliquoted and stored in liquid N₂ in 1 mL aliquots.

Before use, lipids were thawed to room temperature and bath sonicated for 20 seconds three times or until translucent and optically consistent. A 1.5 mL tube was filled with 435 µl of sonicated lipid, 11 µl of Triton-X 100 (to a final concentration of 2.2%, Fisher Scientific, Hampton, NH), 1.3 µl of 0.25 M EDTA (to a final concentration of 0.64 mM, Mallinckrodt Baker, Inc. Paris, KY), protein to the desired concentration, and Buffer K to a final volume of 500 µL. The vesicle solution was gently shaken for 45 minutes with several 5 minute vortex steps during that period.
The vesicles were then added to 50 mg SM-2 Adsorbent Bio-Beads (Bio-Rad Laboratories, Hercules, CA) and gently shaken for at least 8 hours. This was repeated two more times with 75 mg of Bio-Beads for at least 10 hours, and 150 mg of Bio-Beads for at least 8 hours. All quantities were scaled up proportionately for preparations of 800 µL. The vesicles were extruded by making 21 passes through a 200 nm polycarbonate membrane mounted a Lipofast unit (Avestin, Ottawa, ON, Canada) as described in Macdonald et al (25). Mean vesicle diameter, as verified using dynamic light scattering (90Plus Particle Size Analyzer, Brookhaven Instruments Corporation, Holtsville, NY) was 200 nm ± 21.5 nm. All chemicals were from Sigma (Saint Louis, MO) unless otherwise stated.

Proton Flux Assay

The proton flux assay is based on the assay of Schneider and Altendorf (26) as modified by Franklin (12). To measure the flux of protons, 3.84 mL of Translocation Buffer (200 mM Na$_2$SO$_4$, 5 mM MgSO$_4$, and 0.2 mM Tricine titrated to pH 7.00) was stirred for 40-60 minutes to equilibrate temperature and CO$_2$ in the air, thereby reducing pH drift, until the drift was less then ~2 mV per 2 minutes as measured by a highly sensitive micro pH electrode (AccupHast combination electrode model 13-620-297, Fisher Scientific, Hampton, NH) attached to a pH meter (Mettler Toledo, model SevenMulti, Schwärzenbach, Switzerland). Experiments were performed in a water jacketed bath with vial temperature of 24.8 ± 0.8 ºC. The pH, rounded to the thousandths place, was sampled at 2000 Hz, averaged at 4 Hz, and outputted to a computer. Data were exported directly to Excel and saved in a back up data file.
After recording pH from the translocation buffer alone for at least 3 minutes, 3 µL ethanol were added to measure solvent artifacts alone and data were collected for 2 minutes. Vesicles (50 µL) prepared as described were then added to the vial and data were collected for 5 minutes; then 3 µL valinomycin (a potassium ionophore, Sigma, Saint Louis, MO) of a 160 µg/mL stock solution (final concentration 120 ng/mL) were added. After 5 minutes 3 µL carbonyl cyanide m-chlorophenylhydrazone (CCCP, a protonophore, Sigma, Saint Louis, MO) of a 400 µM stock solution (final concentration 300 nM,) were added and after 5 minutes a standard back-titration was performed as follows: addition of 100 nmoles HCl (100 µL of 1 mM HCl), with data collection for 2.5 minutes and a second addition of 100 µL 1 mM HCl, with data collection for 2.5 minutes. An average experiment lasted 20 minutes from ethanol addition to termination of the last back-titration.

Translocation buffer was stored at RT and was stable for 2 – 3 months. Vesicles were stored in the dark at RT and were stable for one month (12). Valinomycin and CCCP were stored at -20°C and were stable for at least 6 months. HCl was stored at RT and was stable for less than one month. Storage for longer times led to a smaller back titration, decreasing our measurement of the buffer strength. Reserve solutions of translocation buffer, valinomycin, CCCP, and HCl were stored at -20 ºC and were stable for at least 6 months. Prior to using any solution, it was allowed to warm completely to room temperature.

The total signal is the final height of the valinomycin plus CCCP signals compared to what would be expected from drift alone. This height is proportional to the trapped volume within all vesicles that did not contain C_L. Determination of the
total signal was as follows. A data trace is printed out from Excel. A straight line is hand drawn through the signal just prior to the valinomycin peak. This line represents the pH drift and is extrapolated forward by hand. The pH drift at the end of the CCCP signal is extrapolated backward by hand. The total signal is then the distance between these two lines taken at one time point, typically the addition of CCCP. The height of the back-titrations is determined in the same manner, i.e. the difference in height between two lines representing the pH drift before and after each addition. The x and y axes of the printouts for all assays from the same day were done on the same scale, i.e. the scales covered the same relative ranges not the same total range.

Results

We had two objectives: 1. to examine the effect of deletion of the *E. coli* gene yraM, which is thought to be a cation-leak (C_L) channel and 2. to use this new strain to quantify the block of proton flux by DCCD. Both of these objectives can be examined with our proton-flux assay. For the second objective we wanted to determine the stoichiometry of inhibition of proton flux by DCCD by determining the proton permeation of the F_O sector of F_OF_1 ATPase.

*Deletion of E. coli yraM gene product C_L*

The identification and deletion of the yraM gene was performed by our collaborator William S. A. Brusilow (Wayne State School of Medicine, Detroit, MI) as describe in the Methods section. He provided us with most of the F_O purified from the deletion and the wild-type strain. Additionally, he provided us with a colony of wild-type and deletion *E. coli* for our own protein preparations, which I learned to do
during a two-week rotation in his laboratory. Some experiments were from \(F_O\) preparations performed in our lab.

We ran a 4 - 20% polyacrylamide gel electrophoresis (PAGE) on the protein from wild-type and deletion *E. coli* to confirm deletion of the yraM gene product (Figure 3-1). The yraM gene product is expected to be about 72 kDa. There are no consistent bands at that weight in the wild-type preparations that are not present in the deletion preparations. Additionally, lane 4 shows a wild-type preparation with a dark doublet at about 40 kDa. In a previous gel this preparation was compared with a deletion preparation, which did not show the doublet, from which we had hoped \(C_L\) is the result of cleavage of the yraM gene product. However, other wild-type preparations, as exemplified by lane 1, do not show the doublet at 40 kDa, suggesting the doublet is not consistent with deletion of \(C_L\).

**Proton Flux Assays**

The protein extract was then reconstituted into lipid vesicles (at pH 7) for analysis in our proton flux assay. Figure 2-2 (Chapter 2) shows the mechanism of proton transport across vesicles. Following addition of valinomycin (a \(K^+\) ionophore) potassium efflux creates a membrane potential of \(-110\) mV inside the vesicles. This potential drives proton influx into vesicles with functional \(F_O\). The proton influx is recorded by measuring the increase in pH of the weakly buffered solution outside the vesicles. The fast rise in pH immediately after valinomycin addition in the vesicles containing \(F_O\) is due to proton movement in response to valinomycin induced \(K^+\) efflux. Proton influx was not detected in protein-free controls after addition of valinomycin. As demistrated in Chapter 2, the slope of the initial valinomycin signal
is proportional to H$^+$ influx. We did observe a small pH drift in most experiments, which was subtracted out by setting it as a baseline for the fitting of the initial slope (Figure 3-2). Addition of the protonophore CCCP serves as a positive control for the presence of vesicles without functional protein, as CCCP allows the exchange of K$^+$ for H$^+$ in all vesicles that did not previously equilibrate K$^+$ for H$^+$. The total signal is defined as the combination of the valinomycin and CCCP signal and is proportional to the total volume entrapped inside the vesicles and depends on the buffer strength inside the vesicles. A standard back-titration of 100 nmoles HCl serves as a calibration for external buffer strength. C$_L$ has been shown by previous researchers (13) to reduce the total signal by allowing potassium to leak out of some vesicles.

Figure 3-2 shows a representative trace of the proton flux assay. The vesicles are stirred for a total of 5 minutes before the addition of valinomycin, including the addition of 3 µl solvent 2 minutes before the valinomycin addition. The pH drift is taken into account by averaging 45 seconds of data points to get a pre-valinomycin slope. The post-valinomycin data are fit by a line through 10 seconds of data starting 3 seconds post-valinomycin. The true valinomycin signal is the difference of the slopes from the pre- and post-valinomycin lines. The ethanol addition shows that ethanol alone causes a small, fast basification of the solution, suggesting that the initial (0-2 seconds) valinomycin signal includes an ethanol artifact.

Figure 3-3 is a close up view of the ethanol and the valinomycin additions in a different experiment than the one visualized in Figure 3-2. The trace in Figure 3-3 is from an experiment will less protein, so the valinomycin signal is smaller, thus enabling easier comparison of the ethanol artifact. Care has been taken during data
analysis to avoid including this artifact in the true proton flux signal observed during the valinomycin signal. It required waiting for a few seconds before data could be collected after the addition of valinomycin to not include the ethanol artifact. The result of this is that in some experiments, particularly ones where the valinomycin signal is not robust such as Figure 3-3, the post-valinomycin slope may be underestimated, resulting in a lower measured flux. Future research using this assay would benefit from elimination of the ethanol artifact by titration of the ethanol, use of another solvent such as DMSO, or the use of curve-fitting programs to predict the true slope.

As mentioned previously, C_l reduces the total signal of the proton flux assay. This assay can therefore be used to detect the activity of C_l and other contaminants. Figure 3-4 shows the results from proton flux assays performed on protein-free vesicles or vesicles made with wild-type or deletion F_o extract. These vesicles were assayed on the same day and made up to the same final concentration of total protein. The total signal is the sum of the valinomycin and the CCCP signal and was measured as described in Methods. As expected, we observed that the deletion yielded a larger total signal than the wild-type. However, the total signals from both vesicle preparations were smaller than that of protein control vesicles. This initial data is consistent with the hypothesis that the gene produce removed in the deletion strain was partly responsible for the decrease in total signal attributed to C_l.

To further quantify the reduction in total signal, the total signal from 40 assays with protein-free controls, 35 assays using wild-type extract, and 64 assays using the deletion extract were measured. With assistance from Center for Collaborative
Research and Statistical Consulting at Brigham Young University, the data were analyzed using the SAS statistical program. It was found that both protein samples showed that the more protein added, the smaller the total signal. However, the difference between the wild-type and deletion preparations was not significant (p < 0.25 as determined by ANOVA). Because there were some contradictory records as to the identity of two protein preparations, these were excluded and the data reanalyzed but the same results were obtained (not significant p < 0.21). We conclude that there is no significant difference between the leakiness of protein extracted from the wild-type and deletion *E. coli* strains.

However, there is always some uncertainty in the protein concentration as determined by the Lowry assay. Even a small global error can make a significant difference in total flux. We are far more confident in the volume of protein aliquoted during a particular vesicle preparation. Therefore, if we take the ratio of two protein concentrations from vesicles made from the same protein batch, any global errors in protein concentration will be eliminated, yielding a more accurate measure of the increase in protein used. Figure 3-5 plots the same data discussed above but using the ratio of the protein concentration versus the ratio of the total signal. The data were normalized for the lowest protein concentration of all experiments from the same protein preparation. In the event of two or more experiments at the same lowest concentration, the data were normalized for the average of the experiments. Taking the ratio reduced our N for wild-type to 27 and for deletion to 49. Although some deletion experiments had a high signal at high protein concentrations, the majority had lower ratios than wild-type. On average, the total signal fell quicker for protein from
the deletion strain than from wild-type. Using ANOVA, this difference was
significant to a p value < 0.02. We conclude we did not eliminate C\textsubscript{L} activity by
eliminating the yraM open reading frame.

*Modeling the Single-Hit Hypothesis*

Previous research (22,23) has shown that while one mole of DCCD per mole
F\textsubscript{O}F\textsubscript{1} ATPase was sufficient to maximally inhibit ATPase activity, two moles of
DCCD per mole F\textsubscript{O} were required to maximally inhibit passive proton flux. A
reduced or partial block of F\textsubscript{O} occurred at 1 mole DCCD per mole F\textsubscript{O}. In a more
recent paper Jones et al., (21) showed that incorporation of only one c subunit with the
mutation of Asp61Gly was sufficient to inhibit F\textsubscript{O} ATPase activity. However,
mutation to the more structurally related Asn required two mutations per F\textsubscript{O} to fully
block passive proton flux.

Since our proton flux assay uses, on average, less than one functional F\textsubscript{O} per
vesicle, it is ideally suited to study the effects of DCCD inhibition at the single
channel level. This assay can be utilized to distinguish between single-hit kinetics and
other models of inhibition. There are three parameters of our assay that will change
upon inhibition of F\textsubscript{O} by DCCD. No matter which model is correct, the total initial
slope, which is the slope of the line fitted to the valinomycin signal, should be
reduced. If the single-hit model is accurate, the slope will be reduced because the
there are fewer functional channel, but each active channel is fully functional. If the
multiple-hit model is accurate the slope will be reduced because most channels are
active, but there is a mix of fully and partially active channels.
Second, the number of functionally incorporated $F_O$ per $\mu g$ total protein assayed will be reduced in the single-hit model, but unaffected in the multiple-hit model. The number of function $F_O$s is calculated from the proportion of vesicles that exhibit proton flux within 5 minutes after addition of valinomycin to the number which equilibrate the membrane charge after addition of CCCP. This ratio is then used to generate a Poisson distribution to determine the number of vesicles that have 0, 1, 2, or more functional $F_O$s. Since the number of vesicles can be determined from the total lipid concentration, the vesicle diameter, and the area per lipid headgroup, the number of total function $F_O$ sectors can be calculated. Under the single hit hypothesis, treatment of $F_O$ with even low concentrations of DCCD will completely inhibit some $F_O$s. Vesicles in which $F_O$ is completely blocked will simply appear to have no functional $F_O$, and in our assay will not exhibit proton flux upon addition of valinomycin. This will increase $P_0$, the number of vesicles with 0 functional $F_O$s. However, if a single hit by DCCD only reduces proton flux by 50%, then all vesicles with $F_O$ hit once should still equilibrate protons over the five minutes before addition of CCCP. However, any $F_O$ hit twice or more times will likely show no proton flux and will only equilibrate protons upon addition of CCCP. Thus, the total number of functionally incorporated $F_O$ in this second case will show a reduced number of functionally incorporated sectors, depending on the number of $F_O$s that are hit multiple times. If numerous different concentrations of DCCD are used this assay could be utilized to generate a Hill coefficient for $F_O$ block. This would be an important contribution to the field and should be an avenue of further research.
Third, the single channel measurement should be unaffected in the single-hit model, but reduced in the multiple-hit model. Using a similar argument as the above paragraph, it can be expected that if F₀ is blocked under a single-hit model, the single channel proton flux measured by this assay will remain the same as with untreated samples. Any unblocked F₀ sectors will pass protons with addition of valinomycin, yielding a high single channel conductance. However, if a single hit of DCCD is only partially effective, a reduction in the single channel flux will be seen.

During the treatment with DCCD and subsequent purification of F₀ from *E. coli* membranes preparation, Dr. Brusilow split the sample into two parts after the membranes had been isolated. One part was treated with DCCD so that the ATP synthase activity was reduced to about 50% of the untreated. The other part was untreated. The membranes were then stripped, F₀ purified, and shipped to our lab in Provo. A second preparation was performed by Dr. Brusilow such that the ATP synthase activity of the treated membrane was reduced to about 80% of the untreated. The proton flux activity of all four protein preparations was assayed at several protein concentrations. Table 3-1 summarizes the values of F₀ activity in moles H⁺ per second per µg protein, number of functionally incorporated F₀S per µg, and the single channel flux in H⁺ per F₀ per second. The standard deviations for each measurement are also reported in Table 3-1.

The single channel flux of F₀ extracted from this new deletion strain is smaller than the 3100 ± 500 H⁺/F₀/sec reported previously by Franklin (12) using the proton flux assay. Although there is the possibility that this represents a true difference in the deletion strain, the difference may be due to the ethanol artifact that required
postponement of data collection for a few seconds after addition of valinomycin. To examine this hypothesis 16 experiments were performed on extracts from the wild-type strain to compare independently with the deletion strain data. The single channel flux was $1910 \pm 1110 \text{H}^+/\text{F}_0/\text{sec}$. The difference between the wild-type and the deletion fluxes was not significant in a one-sided t-test ($p < 0.22$). Therefore, $\text{F}_0$ purified from wild-type and deletion strains of E. coli had statistically similar levels of proton flux. It is likely that the true rate of proton flux lies between 3100 and 1900 $\text{H}^+/\text{F}_0/\text{sec}$.

Table 3-2 summarizes the percent change of activity of the DCCD treated samples as a percent of the paired untreated protein extract and as a percent of the combined untreated extracts. The proton flux of $\text{F}_0$ from the 50% ATP synthase sample shows a reduction to 27% from the untreated sample; while the $\text{F}_0$ activity of the 20% ATP synthase sample shows a 13% reduction. The data suggest that the more DCCD utilized, the greater the reduction in $\text{F}_0$ activity. Further details from both tables are presented graphically in Figure 3-6 through Figure 3-8, which summarize the data relevant to the single-hit hypothesis from the two sets of paired DCCD-treated deletion strain samples.

Figure 3-6 graphically presents the reduction in initial total flux in moles $\text{H}^+$ per second per $\mu$g protein. In the preparation in which 50% of the ATP synthase activity is inhibited (+), the treated $\text{F}_0$ ($N = 9$) shows less total proton flux than untreated $\text{F}_0$ ($N = 2$), showing a reduction to 33% of the untreated. This suggests that DCCD inhibited passive proton flux through $\text{F}_0$. Examining the next preparation treated with more DCCD, the untreated $\text{F}_0$ ($N = 4$) showed a similar activity to the
previous untreated preparation and the treated (++) is reduced to 12% of the untreated
(N=3). The last bar in the graph combines the two untreated preparations (N = 6).
This data suggests that DCCD inhibition is dose dependant.

Figure 3-7 shows the number of functionally incorporated $F_\text{O}$ sectors per $\mu$g
protein used. “Functional $F_\text{O}$” is defined in this assay as any $F_\text{O}$ that can transport
protons at a rate of at least 20% normal (which is the limit of detection for this assay).
This assay did not give consistent results with the lower dose of DCCD having no
apparent effect but the higher dose caused a reduction when compared to either the
paired untreated sample or the combined untreated samples. This reduction is
significant to a $p < 0.038$ in a one-sided t-test. Figure 3-8 presents the single channel
flux, in protons per second per $F_\text{O}$, of the four deletion strain samples treated (or paired
controls) with DCCD (see tables). The two untreated protein purifications show very
similar single channel fluxes. When these two results are combined, untreated $F_\text{O}$
shows a flux of 1610 $H^+ F_\text{O}/sec$ with a standard deviation of 590 $H^+ F_\text{O}/sec$. The 50% ATP synthase inhibited sample (+) has a single channel flux of 455 ± 401 $H^+ F_\text{O}/sec$.
The 80% ATP synthase inhibited sample (++) has a single channel flux of 660 ± 141 $H^+ F_\text{O}/sec$. These represent an activity that is 28% and 41% as great as the combined
untreated samples. Both of the DCCD treated samples show a significantly reduced
flux compared to the untreated samples ($p<0.02$) in a one-sided t-test. This suggests
that the single-hit hypothesis for passive proton flux is not correct for this system.
Discussion

Elimination of the yraM open reading frame

The proton flux assay, as used in our lab, is capable of detecting changes in pH on the order of the mpH scale. Our assay utilizes an efflux of potassium, facilitated by the ionophore valinomycin, to create the membrane potential needed to induce proton influx through individual F_0 sectors of the F_0F_1 ATPase of *E. coli* that are functionally reconstituted into liposomes. The potassium efflux and proton influx continue until the counter-balanced potassium and hydrogen gradients reach a Donnan equilibrium. This dynamic system is modeled and analyzed in Chapter 2. As published by previous researchers from our (12) the Poisson distribution can be used to deduce the number of function F_0 channels per vesicle. Using dynamic light scattering and the lipid concentration we can quantify how many vesicles are present per experiment. Single channel conductance of F_0 can then be determined based on the total hydrogen flux upon addition of valinomycin.

With recent advances in genetic sequencing, the genomes of many species have been entirely sequenced including the bacterium *Escherichia coli*. These DNA sequences reveal many open reading frames, which are suspected to encode proteins, but for which no protein has been reported. The *E. coli* yraM gene (GenBank accession number AAC76181), is one such sequence. While studying proton flux through F_0 Cao et al. (13) discovered a channel that co-purifies with F_0 and that leaks potassium, which was named cation-leak channel (C_L). Our collaborator William Brusilow and co-workers (Wayne State University School of Medicine, Detroit, MI) attempted to isolated this channel and by sequence analysis deduced it was the yraM
gene product. They then created a knock-out strain, from which $F_O$ has been purified and its proton flux determined and compared to the wild-type strain.

Cao et al. (13) showed that during proton flux assays, the total signal (magnitude of pH change following addition of both valinomycin and CCCP) was reduced when “too much” protein was added. This loss in signal was attributed to $C_L$ eliminating the potassium gradient in some proteoliposomes, thereby rendering them unresponsive to the addition of valinomycin. It was also shown that heat treatment of the proteoliposomes removes both proton flux activity and this leak activity, suggesting that the lead is indeed due to a protein. In his dissertation Dr. Franklin (27), who also used the proton flux assay to study $F_O$, showed that the reduction in total signal was dependent on the final concentration of total protein.

If $C_L$ were the yraM product, it was expected that $F_O$ purified from the strain with the yraM gene deleted should show a greater total signal than $F_O$ purified from the wild-type strain. Initial results (Figure 3-4) seemed to confirm this hypothesis. Vesicles made with the wild-type extract have a smaller total signal than vesicles made with the deletion extract. This difference was attributed to the presence of $C_L$. $F_O$ purified from the deletion strain showed a larger total signal than wild-type, but less than control. This suggests that $C_L$ activity had multiple components, one of which was eliminated in the deletion strain. The fact that $C_L$ does not appear to be totally lost with the removal of the yraM gene does not preclude it from being the yraM gene produce. There may be other contaminants that co-purify with $F_O$ and that leak cations besides $C_L$. This latter hypothesis seems more likely the case since the
deletion showed a marked improvement in total signal (3.6x larger) compared to wild-type. This argues that $C_L$ is the major contaminant cation channel.

However, upon repeating the assay for a total of 35 wild-type assays and 64 deletion assays over many protein concentrations the data argue that $C_L$ activity was not deleted. Figure 3-5 shows the results after normalizing the protein concentration for each assay from the same protein preparation. The exponential fit of the total signal of the deletion samples fall more quickly than that of the wild-type samples. This difference is significant to a $p$ value $< 0.021$ (as determined by ANOVA). These data suggest that deletion of the yraM open reading frame actually made the cation-leak worse. This leads to the question of what the yraM gene product really is.

A possible clue to the role of the yraM gene product is the observation that the deletion strain grew quicker than the wild-type strain. Let’s assume that yraM does encode for one of the constituents of $C_L$ and another constituents is a metabolic waste product or a protein essential for growth. If the wild-type and the deletion strains are in different stages of growth it is possible that the decrease in total signal in deletion may be due to a build up of the other constituent. This hypothesis can be tested by separating the data according to the extent of growth in each preparation, as measured by OD at the time of harvest. The ratio of the total signal was then examined according to differences in growth. As presented in Figure 3-5, the average of all samples shows a negative correlation (downward decay) of total signal as a function of protein. However, in the deletion strain, the higher OD prep ($\sim$OD 2) was flat with protein concentration, i.e. addition of more protein did not decrease the total signal based on 9 experiments. The lower OD ($\sim$OD 1.3) $F_0$ preparations from deletion
strain (N = 8) and all the wild type preparations had a negative correlation with protein concentration. Specifically, in the wild-type strain the high OD (OD 4, N = 3) showed a negative correlation with protein concentration, as did the low OD (OD 1.3 - 1.7, N = 18), but the high OD cells had less total signal than the low OD cells. From these data we can conclude that growth conditions may have an effect on total signal. If they do, one component of C₅ may likely be involved in growth or a metabolic waste. Further studies on the dependence of total signal on the growth conditions may clarify new roles of C₅ and the yraM open reading frame.

The most convincing evidence that the yraM open reading frame expresses the C₅ channel would be to put the open reading frame into an *E. coli* expression vector, express the gene product, and study the channel-like properties using electrophysiology (e.g., a bilayer set-up). These properties could then be compared with the properties of C₅, which have been reported by Cao et al. (13). Confirmation of cation-like channel properties with the purified yraM gene product would be conclusive evidence it is the C₅ gene. Such studies are suggested to future researchers.

*Model for Inhibition of F₀ by DCCD*

Fillingame et al. (17,18,19,20,21) have suggested that one molecule of DCCD is sufficient to inhibit passive proton flux through an individual F₀ sector. Our assay has the sensitivity to detect proton flux through F₀ in response to a voltage gradient at the single channel level. It also has the ability to distinguish vesicles with zero functional F₀ sectors reconstituted from vesicles with at least one partially active
channel. As described in Results, these aspects have been used to model how our assay would be affected under the assumption of single-hit kinetics.

As expected for both the single-hit and multiple-hit models, the total initial slope decreased with treatment of DCCD (Figure 3-6). This response appears to be dose-dependent. Figure 3-7 shows that only the higher treatment with DCCD had any affect on the number of function $F_O$ channels per $\mu$g protein. This supports the two-hit model for passive proton flux based on the following. If ATP synthase activity is inhibited by one DCCD per $F_O$, then at least 80% $F_O$ were hit at least once with DCCD. Using the Poisson distribution 50% $F_O$ were hit at least twice and therefore totally inhibited. Therefore we expected a 50% reduction in the number of functional $F_O$. The difference between the uninhibited and the inhibited are different from a 50% reduction. These data suggest that the two-hit model is correct (but not a four-hit model). In the sample treated with less DCCD, using a similar argument we expected only a 16% reduction in the number of functional $F_O$. Our assay is not sensitive enough to resolve such a small reduction.

The most conclusive evidence for a multiple-hit model is seen in Figure 3-8, which shows a reduction in the calculated single channel flux with treatment of DCCD. If the single-hit model accurately described passive proton flux, then this number would be unaffected by treatment with DCCD because any vesicles containing $F_O$ that was hit by even one DCCD would act like protein-free vesicles while any un-hit vesicles would exhibit normal proton flux. In our experiments only a few percent of vesicles had multiple copies of $F_O$, so they would not noticeably reduce the single channel flux in a single-hit scenario. However, the data show that there a significant
reduction (both $p < 0.02$ in a single-sided t-test) in single channel flux, suggesting that addition of DCCD in single-hit stoichiometries causes $F_0$ to passively transport protons at a lower but non-zero rate.

There are three key findings of these studies. First a cation-leak channel $C_L$, which in our assay reduces the total signal, was not sufficiently linked to the gene product of the open yraM reading frame. Measurements of the total signal of $F_0$ isolated from wild-type and deletion strains did not show a significant recovery of signal and therefore we cannot confirm identification of the yraM gene product as $C_L$, but the gene may alter cell growth. Second, $F_0$ isolated from this new deletion strain exhibits similar activity as $F_0$ isolated from the wild-type strain. Third, using several parameters of the proton flux assay it was determined that the inhibition of $F_0$ by DCCD is more complex than the single-hit hypothesis. It appears that a single-hit reduces, but does not eliminate proton flux through $F_0$. The data are consistent with a two-hit model.
Figure 3-1 – Comparison of protein isolated during F₀ extraction from wild type and deletion E. coli strains. The lanes contain F₀ purified from either wild-type or deletion E. coli, as labeled at the top. Lane 7 is a molecular weight standard. Lane 4 shows a wild-type preparation with a dark double at about 40 kDa, which is not present in the other wild-type preparation (lane 1).
Figure 3-2 – Representative pH tracing of a proton flux assay. Vesicles were added at time zero (not shown). Ethanol, the solvent for valinomycin and CCCP, was added after 3 minutes (diamond). Valinomycin, a potassium ionophore, induces a membrane potential at 5 minutes (arrow). CCCP, a protonophore, was added at 10 minutes, serving as a positive control for vesicles without functional $F_0$ (arrowhead). Two additions of 100 nmoles of HCl serve as a standard calibration for buffer strength. The line through the valinomycin addition shows the fit of the initial slope to the pH curve after addition of valinomycin, as described in Methods. This slope of the line after valinomycin addition is corrected by taking into account the slope of the pH drift (not shown).
Figure 3-3 – pH trace emphasizing the ethanol artifact from a different experiment than Figure 3-2. Addition of ethanol is shown by the diamond and addition of valinomycin (in ethanol) is shown by the arrow.
Figure 3-4 – Total signal from F₀ purified from wild-type and deletion strains versus a protein-free control. The same concentration of total protein was assayed on the same day. Vesicle diameters were similar in all three preparations. The greater total signal in vesicles containing F₀ purified from the deletion strain suggest that deletion of the yraM open reading frame is responsible for the larger signal. The fact that the total signal of the deletion does not match control, suggests that a second leak may also contribute to K⁺ leak in reconstituted vesicles. Addition of valinomycin is shown by the arrow and the addition of CCCP is shown by the arrowhead.
Figure 3-5 – Plot of the ratio of total signal of F₀ isolated from wild-type and deletion E. coli strains versus the ratio of concentration of reconstituted protein. The plot shows total signal as a function of total protein isolate (in µg protein in the reaction vial).

The trend lines are exponential fits. The difference between these the exponential fits (as determined by ANOVA) is significant to $p < 0.021$. 
Table 3-1 – Results of the proton flux assay in four preparations. One in which the ATP synthase activity is reduced by ~50%, one in which it is reduced by 80%, and the parallel, but untreated purifications. Also shown are the data when the results from the two untreated samples are pooled.

<table>
<thead>
<tr>
<th></th>
<th>Mol H+/Sec/µg</th>
<th>Stdev</th>
<th>Functional Fo/µg</th>
<th>Stdev</th>
<th>Single Channel Flux</th>
<th>Stdev</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>8.97E-11</td>
<td>3.52E-11</td>
<td>3.3E+10</td>
<td>1.76E+10</td>
<td>1708</td>
<td>269</td>
<td>2</td>
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<tr>
<td>50% ATP Synthase</td>
<td>2.93E-11</td>
<td>2.66E-11</td>
<td>3.8E+10</td>
<td>2.06E+10</td>
<td>455</td>
<td>401</td>
<td>9</td>
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<tr>
<td>Untreated</td>
<td>1.17E-10</td>
<td>2.72E-11</td>
<td>5.5E+10</td>
<td>3.08E+10</td>
<td>1555</td>
<td>734</td>
<td>4</td>
</tr>
<tr>
<td>20% ATP Synthase</td>
<td>1.37E-11</td>
<td>2.04E-12</td>
<td>1.3E+10</td>
<td>4.13E+09</td>
<td>660</td>
<td>141</td>
<td>3</td>
</tr>
<tr>
<td>Both Untreated</td>
<td>1.08E-10</td>
<td>2.99E-11</td>
<td>4.7E+10</td>
<td>2.75E+10</td>
<td>1606</td>
<td>586</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>% Fo Activity</td>
<td>Untreated Combined</td>
<td>% Incorporation</td>
<td>Untreated Combined</td>
<td>% Single Channel</td>
<td>Untreated Combined</td>
<td></td>
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<tr>
<td>------------------</td>
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<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>100%</td>
<td>100%</td>
<td></td>
<td></td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50% ATP Synthase</td>
<td>33%</td>
<td>27%</td>
<td>114%</td>
<td>79%</td>
<td>27%</td>
<td>28%</td>
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<tr>
<td>Untreated</td>
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<td>100%</td>
<td></td>
<td></td>
<td>100%</td>
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</tr>
<tr>
<td>20% ATP Synthase</td>
<td>12%</td>
<td>13%</td>
<td>24%</td>
<td>27%</td>
<td>42%</td>
<td>41%</td>
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</table>

**Table 3-2** – Change in the response of the proton flux assay to DCCD treatment. The percent change is also shown when the results from the two untreated preparations are combined.
Figure 3-6 – The initial total proton uptake through $F_0$ is altered by the addition of DCCD (+ = low dose, ++ = high dose). Black bars to the left of each treated sample are the untreated paired controls. The right bar is the average of both untreated samples. $F_0$ activity is reported as moles H$^+$/second/µg protein. The error bars show one standard error in this and all subsequent charts.
Figure 3-7 – The number of functionally reconstituted F₀ channels per µg protein.

Most of the preparations provide similar numbers of active channels after purification and reconstitution, although treatment with the highest amount of DCCD, which caused 80% ATP synthase inhibition (++), reduced the number of functional F₀₅s. Since these samples are all from deletion strain; the number of F₀₅ per µg protein incorporated from all wild-type (WT) preparations (N = 20) is also shown for comparison.
Figure 3-8 – Calculation of the single channel flux. Fo treated with DCCD shows a significant reduction in proton conductance. These reductions are significant to a p<0.02 in a single sided t-test. See text for further discussion; columns are same as in Figure 3-6.
Reference List


Chapter 4 – Concluding Remarks

Protons and proton gradients play a number of crucial roles in homeostasis of organisms. In the life cycle of the Influenza A virus the proton channel M2 is the key player responsible for sensing the right conditions to initiate viral uncoating via a pH sensitive gate, the first such mechanism suggested. Once activated, M2 passes protons into the virus capsid which triggers viral replication. In the synthesis of ATP almost all living species use some form of the $F_0F_1$ ATPase. This enzyme turns a pre-established proton gradient (PMF) into rotary motion, driving the reaction:

$$ADP + P_i \rightarrow ATP.$$  

This thesis utilized an assay where a $K^+$ gradient-induced membrane potential drove proton influx into vesicles, thereby detecting the activity of functionally reconstituted M2 or $F_0$ channels. The results of the proton flux activity were examined to discriminate useful information about channel function and inhibition.

Three key features of M2 function were clarified during the course of our studies. The acid-activation of M2 has often been claimed based on a relatively small (2 – 10 fold) increase in channel conductance in the presence of a large change in $H^+$ concentration (30 – 100 fold). However, a large change in conductance is expected due solely to the increased presence of $H^+$ in the bath at lower pH. The fact that there is little or no increase in conductance at more acidic pHs suggests that some step in the translocation process, perhaps a titratable site within the conduction pathway, is already saturated by pH 7. This site was postulated to be histidine-37. Also, Chapter 2 shows that the block of M2 by amantadine is reduced at pH 5.4. Finally, previous studies using assays similar to ours have concluded that M2 and other proteins are
highly selective to protons compared to other cations. Using a mathematical model created by Dr. Busath in conjunction with experiments using gramicidin, a mildly selective ion channel, it was shown that selectivity for hydrogen only need be a factor of about 10 times that of potassium to observe a valinomycin-induced proton signal. In contrast to gramicidin, it was determined in this study that M2 is $10^7$ times selective for protons over potassium.

Three key findings of using our proton flux assay on $F_o$ extracts from *E. coli* were discovered together with our collaborator, Dr. Brusilow. First a cation-leak channel $C_L$, which in our assay reduces the total signal, was potentially linked to the gene product of the open yraM reading frame. A strain was created in which the yraM gene was deleted from the *E. coli* genome. Measurements of the total signal from $F_o$ isolated from wild type and deletion strains did not show a significant recovery of signal and therefore we can not confirm identification of the yraM gene product as $C_L$. However, differences in growth rates of the wild-type and the deletion strain suggest that other contaminating channels may also reduce the total signal in our proton flux assay. It was established that $F_o$ isolated from this new deletion strain exhibits similar activity as $F_o$ isolated from the wild-type strain. Finally, it is known that DCCD blocks proton flux through $F_o$ by binding to the c-subunit ring responsible for proton translocation. However, the stoichiometry of this inhibition has been a matter of debate in the literature and is often reported to be one DCCD per $F_o$. Using several parameters of the proton flux assay it was determined here that the inhibition of $F_o$ by DCCD is more complex than the single-hit hypothesis. It appears that a single hit (one DCCD binding to one c subunit of the c-ring) reduces, but does not eliminate passive
proton flux through F\textsubscript{0}. The data are consistent with a two-hit model but not a four or more hit model.

In both studies one essential aspect of determining the flux per channel was the estimation of the number of active channels. In the M2 studies, the nominal protein concentration (40.5 µg at pH 5.4 and 15 µg at pH 7.0) was used with the assumption that the preparation contained no contaminating channels and that all the M2 was fully functional. In the F\textsubscript{0} studies the Poisson distribution was utilized, assuming that any functional F\textsubscript{0} had no gating and would fully equilibrate the vesicular membrane potential during 5 minutes after addition of valinomycin. The average number of functional F\textsubscript{0} channels was 6x10\textsuperscript{11} in the DCCD studies. If we use the nominal total protein concentration of 21.4 µg, the average protein concentration in the DCCD experiments, to calculate the expected number of functional F\textsubscript{0} channels we expect 8.1 or 9.5x10\textsuperscript{13} functional F\textsubscript{0} channels (containing 9 or 12 c subunits, respectively).

Using the nominal protein density greatly overestimates the number of functional F\textsubscript{0}s (by ~150X). This was expected since the protein preparation is far from pure (See Figure 3-1). However, the M2 preparations are much purer due to the use of a His-tag during purification. The M2 gel (See Figure 2-1) shows M2 to be the major protein purified. Therefore the nominal protein calculation used in Chapter 2 should be correct within a factor of 2 – 5. This would mean that M2 may conduct protons at a rate of as high as 1 – 2.5 H\textsuperscript{+}/tetramer/sec at pH 7.

Although the mechanisms of proton transport through M2 and F\textsubscript{0} are quite different, both channels conduct protons: M2 at 0.5 H\textsuperscript{+}/tetramer/second and F\textsubscript{0} at 1600 H\textsuperscript{+}/F\textsubscript{0}/second. Therefore the time-averaged proton flux of F\textsubscript{0} is ~3200 times that of
M2. However, if M2 is only in the open state 1/1000th of the time, both channels conduct protons equally well in the open state. The idea that Fo can continuously conduct at such a rate without stalling out is intriguing. Protons must be delivered to the mouth of the pore at least that quickly. Lipid headgroup buffering may play a role in recruiting protons to the pore, but even at pH 7 we are above the pKₐ of PE and PG, the major components of the E. coli lipid extract used in Fo reconstitution (1). More likely simple diffusion of H⁺ through water or H⁺ transported by the buffer to the pore is sufficient to supply the necessary protons. In any event, the Fo proton current measured in this study (on the order of 1 pS) is far less than the 300 pS to 1000 pS reported for gramicidin at pH 7 by Cukierman (2).

It is possible our observed proton flux was limited by valinomycin transport of K⁺, but this rate has been shown to be 1₀⁴ per second (3) in a 1 M potassium solution and enough valinomycin was added so that each vesicle contained many molecules. Thus, the rate of proton transport through Fo was orders of magnitude smaller than the valinomycin transport of potassium, as confirmed in our experimental observation that our proton flux did not increase when the amount of valinomycin was doubled or when valinomycin was added twice.

Numerous avenues are now open for further research of proton conduction in both M2 and Fo. In the M2 project there are many parameters that are yet to be studied, such as dose response curves using different concentrations of M2 and of amantadine, internalization of amantadine, reconstituting M2 by various methods such as dialysis, more thoroughly measuring proton flux with changes in pH for studying acid activation and His37 titration, and evaluation of M2 protein orientation in smaller
vesicles. It is highly recommended to attempt the pH 9 studies using a different buffer species, particularly a non-organic species that is not lipid permeable.

In the F\textsubscript{O} project the confirmation of the cation-like channel properties of an expression construct containing the yraM gene would be the most conclusive evidence to show that it is the \( C_L \) gene (if it is). Future research using the proton flux assay would benefit from elimination of the ethanol artifact, which has reduced the sensitivity of the current assay by causing a small pH jump upon addition of valinomycin. This may be accomplished by titration of the ethanol with concentrated HCl. Conversely, the ethanol artifact may be eliminated by dissolving valinomycin in DMSO a non-titratable solvent. If the artifact can not be eliminated, the use of nonlinear curve fitting, may allow for a more careful determination of the \( F_O \) proton flux rate. This could be accomplished by modifying the Excel spreadsheet I created to fit the initial data to an exponential curve rather than a line or it could be accomplished with the help of Dr. Barry Willardson in the Chemistry Department, who uses a different program to fit data curves. Finally, the determination of the Hill coefficient of the stoichiometry of DCCD inhibition of passive proton flux through \( F_O \) has not been determined. Our assay has the unique possibility of determining this coefficient on a single channel level. This would serve as the final essential piece of evidence supporting or refuting the single-hit hypothesis.
Reference List


EDUCATION
B.S. August 2004 Brigham Young University Major: Zoology (Human Biology emphasis).
M.S. August 2006 Brigham Young University Major: Physiology.
M.D. Accepted for Class of 2010 University of Utah School of Medicine.

SCHOLASTIC ACHIEVEMENTS
Publications

J. C. Moffat, et al. “Identification and Deletion of a Previously Undescribed E. coli Gene Product that Co-purifies with F₀.” *Biophysical Journal* 90:283a. 2006. (This poster presentation won the Student Research Achievement Awards (SRAA) Poster Competition for the Transport/Permeation subgroup at the 206 Biophysical Society meeting.)


Research and Teaching Assistantships
6/02 – Present Research Assistant with Dr. Woodbury studying F-Type ATP Synthase and with Dr. Busath studying M2 channel from the Influenza A virus.
8/04 – 4/05 Teaching assistant for physiology lab.
1/03 – 4/03 Teaching assistant for organic chemistry lab.

Membership in Professional Societies
Biophysical Society and Sigma Xi
HONORS RECEIVED

Scholarships
Fall 2003 Physiology and Develop. Bio. Department Undergraduate Scholarship.
Summer 2003 BYU Academic Half Tuition Scholarship.

Other Awards
Feb 2006 Student Research Achievement Awards (SRAA) Poster Competition for the Transport/Permeation subgroup at the 2006 Biophysical Society meeting.
Feb 2005 BYU Research Presentation Award for 2005 presentation at Biophysical Soc.
April 2000 Presidential Award for my volunteer efforts with my church in Philadelphia.
April 1995 Rank of Eagle Scout awarded.

EXTRA-CURRICULAR ACTIVITIES
Volunteering
7/05 – 4/06 Trauma I Emergency Room Twice Month where I assist in patient care.
5/05 – 7/05 Translated Spanish for a physician assistant at a community health clinic.
11/02 – 12/05 Farrer Elementary tutoring children in reading and writing.
5/02 – 8/02 Ear clinic helping prepare presentation slides for medical professionals.
5/98 – 5/00 Volunteered 205 hours in Thomas Jefferson Hospital in Philadelphia.
12/98 – 2/99 Parkview Hospital in physical therapy lab where I talked with patients.
6/98 – 5/00 Volunteered 80 hours a week for my church in Philadelphia - learned Spanish.

OTHER SIGNIFICANT ACCOMPLISHMENTS
Leadership Opportunities
8/04-Present Team leader of a research group where I mentored 6 undergraduate students.
9/04 – 9/05 Secretary in the men’s organization of my local congregation.
8/01 – 12/01 Student Supervisor at work in a busy cafeteria during dinner shift.
9/97 – 4/00 BYU Student Leadership Seminar. Participant Fall ’97, Winter ’98 and Fall ’00. Fellow in Winter ‘01. As a fellow I led discussions on leadership topics.

School and Summer Work
In addition to the above assistantships I have supported myself during school by working:
10/00 – 1/02 BYU Dining Services student cook and shift supervisor.
8/00 – 10/00 Early morning custodian.