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Philosophy of voltage-gated proton channels

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In this review, voltage-gated proton channels are considered from a mainly teleological perspective. Why do proton channels exist? What good are they? Why did they go to such lengths to develop several unique hallmark properties such as extreme selectivity and ΔpH-dependent gating? Why is their current so minuscule? How do they manage to be so selective? What is the basis for our belief that they conduct H^+ and not OH^-? Why do they exist in many species as dimers when the monomeric form seems to work quite well? It is hoped that pondering these questions will provide an introduction to these channels and a way to logically organize their peculiar properties as well as to understand how they are able to carry out some of their better-established biological functions.

1. Introduction

Voltage-gated proton channels (H_V) are membrane proteins that mediate the rapid movement of protons (H^+) across cell membranes while excluding all other ions. Like other ion channels, the proton channel adopts two functional configurations. When 'open', each channel allows up to $10^5\,H^+$ to cross the membrane per second but when they are 'closed', they do not allow any ions to cross the membrane. They are 'voltage-gated' because they open or close in response to changes in the membrane potential of the cell. Because H^+ carries a positive charge, its movement across the membrane comprises an electrical current that can be recorded experimentally. A voltage-gated proton channel gene was not identified until 2006 [1,2]. Since then, some of the working parts of the molecule have been described, for example the conduction pathway (figure 1), but no crystal structure exists. Although a large number of enzymes, including many crucial to bioenergetics, contain proton pathways (or channels) that are essential to the operation of the molecule, the voltage-gated proton channel is a distinct category of protein and has not been reported to exist in mitochondria or chloroplasts. In many cells, proton channels are present in the plasma membrane; in a few instances (dinoflagellates and phagosomes in white blood cells), they have been shown to exist in organelle membranes.

When these words were written, this was the 100th review written entirely ($n = 75$) or partially ($n = 25$) about voltage-gated proton channels. The number of original research papers, 157, was less than double that number[1]. From these statistics, one might conclude that this review needs only to discuss the two most recent original papers. Instead, we will step back and attempt to view the subject from a distance. Why do voltage-gated proton channels exist? What good are they? Why do they have so many unique properties? What kind of a world would it be if there were no proton channels? We describe the properties of proton channels teleologically and anthropomorphically; we do not pretend to understand the evolutionary forces that produced this protein, but simply describe it as though it were a sentient being whose properties serve the purposes we impute to it.

1.1. The origin of H_V species

The abbreviation for voltage-gated proton channels is H_V (H for H^+, the conducted ionic species, subscript V for voltage-gated). To date no more than

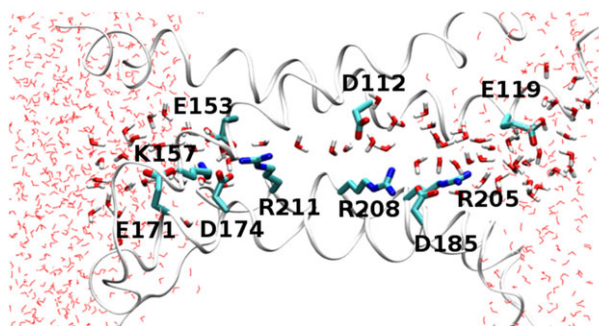© 2013 The Author(s) Published by the Royal Society. All rights reserved.

Figure 1. A snapshot from a molecular dynamics simulation of the open state of the human voltage-gated proton channel, hH$_V$1. The four transmembrane helices are shown as ribbons with the extracellular end of the channel to the right. Charged amino acids (labelled) and water molecules in the pore are shown in licorice representation. This snapshot illustrates a configuration that is present only 10% of the time, in which there is a continuous water wire; usually the water wire is interrupted by a D112 – R208 salt bridge. The external and internal charge networks are thought to stabilize the open conformation of the channel. (Adapted from [3].)

one gene has been identified in any species, thus all H$_V$ are H$_V$1, with a prefix that indicates species (e.g. hH$_V$1 for human, EhH$_V$1 for the coccolithophore *Emiliania huxleyi*). The official numbering of other channels is often more complex, including subtypes, e.g. K$_V$3.2. The H$_V$ family tree (figure 2) continues to grow, but already includes several kingdoms (or supergroups, if one prefers this classification system), including unconfirmed H$_V$ in *Chlorella* (Protista kingdom) and in the common wine grape, *Vitis vinifera* (Plantae kingdom), confirming the Bacchanalian proclivities of H$_V$. Taylor *et al*. [5] noted that H$_V$ appear to be absent in land plants and suggested an explanation: these species tend to have a large inward pH gradient, which seems fundamentally incompatible with the exquisite design of the proton channel (in most species) as a passive acid extrusion mechanism. This argument may apply to plasma membranes, but H$_V$ could still exist in plant vacuole membranes. Recent BLAST searches produce a handful of likely candidates among algae, fungi and a few higher plants. The branch length in figure 2 reflects the extent of difference in amino acid sequence between the branch-dweller and its neighbours. Mammalian H$_V$1 are all very similar to each other, but as we branch out to invertebrates, diversity blossoms and single-celled organisms have wildly diverse sequences. Sequence differences, in turn, might be expected to presage functional differences, nicely illustrated by the novel properties of the kH$_V$1 in the dinoflagellate *Karlodinium veneficum* [4], which has the longest branch of all. This is the only known proton channel that opens to allow inward proton current; all other H$_V$ open only when there is an outward electrochemical proton gradient, and consequently their predominant function is acid extrusion from cells (see 'Why do proton channels have ΔpH-dependent gating?'). Because kH$_V$1 opens negative to the Nernst potential for protons, E_H, it allows inward current, and is thus capable of mediating an action potential [4], just as are voltage-gated sodium channels in more traditional excitable cells such as nerve and muscle [6]. As the H$_V$ family tree grows, and especially as the properties of H$_V$ in the 30 not-yet-studied species in figure 2 are explored, further diversity in function will likely be found.

Based on sequence homology analysis [4,7], H$_V$ are closely related to two other groups of proteins that also contain voltage-sensing domains (VSDs), the voltage-sensing

phosphatases (VSP) and c15orf27 (molecules of unknown function). These groups are in turn related by common ancestry to Na$^+$ and Ca^{2+} channels, and appear to have diverged from the K$^+$ channel family over one billion years ago [3]. Figure 3 illustrates the molecular structure and membrane topology of three classes of VSD-containing molecules.

1.2. Why do voltage-gated proton channels exist?

A flippant answer to this question is 'for the same reason that other ion channels exist!' Cells need to regulate the flow of ions across their membranes, and the most sensible way of doing this is to produce an array of proteins that selectively transport individual ionic species in a tightly controlled fashion. Because the consequences of allowing ions to cross membranes differ drastically for each species of ion, we must delve a bit deeper for a more informative answer. Reflecting the importance of the conducted ion species, traditionally ion channels have received names that indicate the type of ion they transport, sometimes modified by a salient characteristic (inwardly rectifying K$^+$ channel; voltage-gated Na$^+$ channel; Ca^{2+}-activated K$^+$ channel, stretch-activated non-selective cation channel). When a channel opens in a cell membrane, it conducts ions passively down the electrochemical gradient for the conducted ion. The chemical part of an electrochemical gradient simply reflects that the concentration of one ionic species is often greater on one side of the membrane. Ions tend to flow down the gradient, from high to low concentration, other things being equal ('other things' being the electrical contribution discussed in the next paragraph). Usually K$^+$ current is outward, because the K$^+$ concentration is much greater inside the cell, [K$^+$]$_i$, than outside, [K$^+$]$_o$. Most cells have large inward Na$^+$ and Ca^{2+} gradients, meaning that the concentrations of these cations are much higher outside the cell than inside; hence Na$^+$ and Ca^{2+} channels almost always carry inward current.

1.2.1. A digression on the Nernst potential

In addition to the chemical gradient, we also need to consider the electrical potential across the cell membrane, the 'membrane potential'. Because ions are charged, they are affected by electrical fields. For example, [K$^+$] is approximately 160 mM inside cells and 4.5 mM outside, so in the absence of electrical forces, a K$^+$ channel would carry outward current. Electrical current is defined in terms of the movement of positive charge, so this means K$^+$ leaves the cell. This definition makes the behaviour of anions less intuitively accessible; outward Cl$^-$ current is recorded electrically when there is inward Cl$^-$ flux ('flux' simply means the physical movement of ions from one place to another). The effects of a concentration gradient can be modified or overridden if we control the membrane potential, which can be done experimentally by means of a circuit called a 'voltage clamp'. Membrane potential is defined as the potential inside the cell relative to outside, which is defined as the reference voltage, 0 mV. As the membrane is hyperpolarized[2], the increasingly negative charge inside the cell tends to prevent the positively charged K$^+$ from leaving, despite the outward concentration gradient. We eventually reach a special voltage called the Nernst potential for K$^+$, E_K, at which the electrical force perfectly balances the chemical potential and no net current will flow:

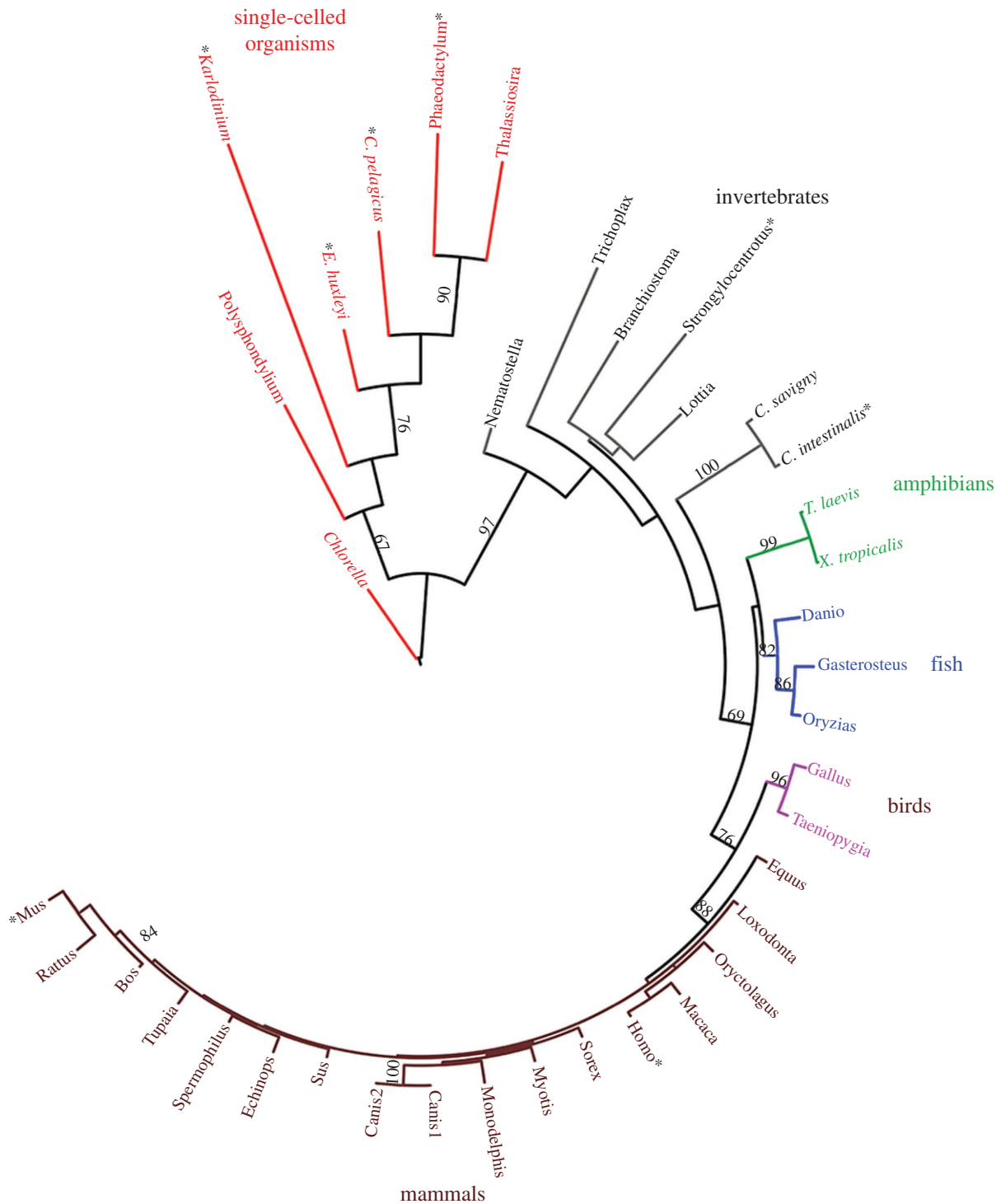$$E_K = 2.303 \frac{RT}{zF} \log \frac{[K^+]_o}{[K^+]_i}, \quad (1.1)$$

Figure 2. Phylogenetic tree of confirmed and probable voltage-gated proton channels. Branch length is proportional to the degree of difference from neighbouring proteins. For the eight starred species, the gene has been expressed heterologously and confirmed electrophysiologically to encode a voltage-gated proton channel. (Adapted from [4].)

where R is the gas constant, T is temperature, F is Faraday's constant, z is the charge of the ion (+1 in this case), $[K^+]_o$ is the extracellular (outside) concentration of K^+, and $[K^+]_i$ is the intracellular concentration of K^+. The constant $2.303(RT/zF)$ is approximately 58 mV at 20°C so when $[K^+]_o = [K^+]_i$, $E_K = 0$ mV and when there is a 10-fold outward K^+ gradient, e.g. $[K^+]_i = 160$ mM and $[K^+]_o = 16$ mM, E_K is −58 mV. The Nernst potentials for other ions are calculated similarly, with the *caveat* that the correct z must be used for anions or divalent ions. If we consider our favourite channel, the voltage-gated proton channel, its permeant ion is H^+ alone. The concentration of H^+ is traditionally presented as

pH, the negative logarithm of the concentration, so 10^{-7} M H^+ is pH 7. We define the pH gradient ΔpH as $pH_o - pH_i$, and from the Nernst equation (equation (1.1)), we see that for $\Delta pH = 1$ unit, E_H is −58 mV. A positive ΔpH means there is an outward H^+ (chemical) gradient; which means that at 0 mV (no applied voltage), H^+ will tend to exit the cell through any H^+ selective pathway. The Nernst potential is very useful in characterizing ion channels. The selectivity of a channel (which ions it allows to pass) can be determined by varying ion concentrations and measuring the reversal potential (the voltage where no net current flows), and comparing this to the Nernst potential calculated for each ionic species present.
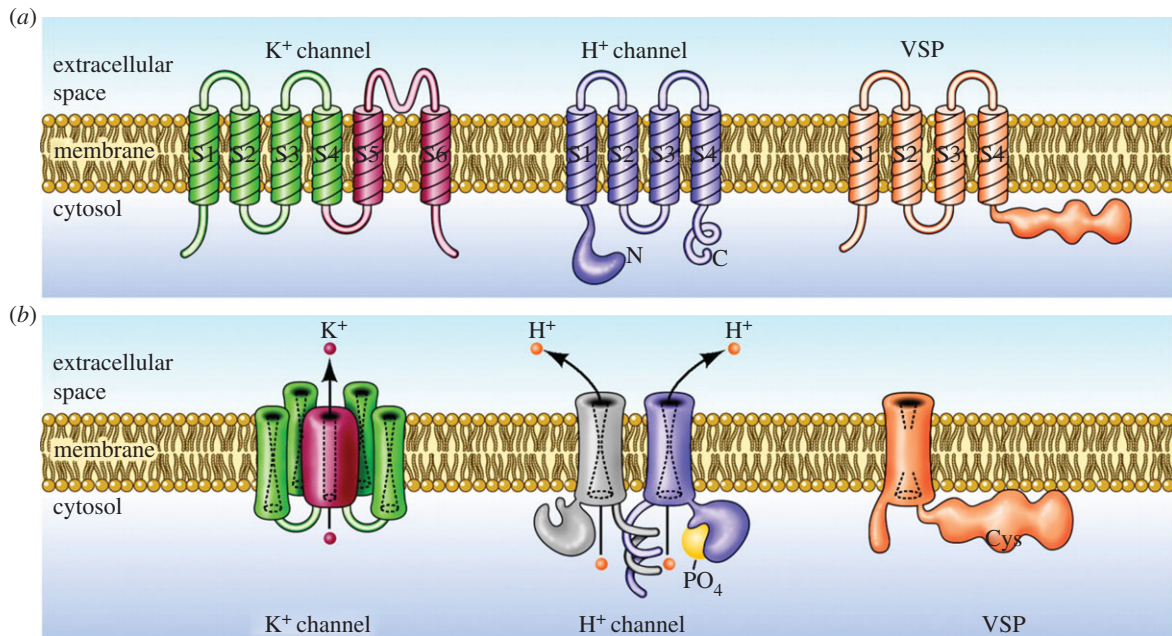
Figure 3. Molecular architecture and orientation in the membrane of three classes of molecules that contain VSDs. (*a*) Illustrates the monomer of each, with its transmembrane segments numbered (S1, S2, etc.) starting from the intracellular N terminus. In each, the VSD includes the first four segments, S1–S4, in particular S4 which contains a series of cationic residues (Arg or Lys) that are thought to move outward upon membrane depolarization. The VSDs from these molecules are homologous. (*b*) Each molecule in its functional oligomeric state. The voltage-gated potassium (K$^+$) channel is a tetramer, with four VSDs surrounding a single central pore, formed by the four S5–S6 segments, through which K$^+$ crosses the membrane. The proton channel is a dimer in mammals and many other species, but each monomer has its own conduction pathway [8,9] and can function independently. Phosphorylation of Thr^{29} in the intracellular N terminus enhances the gating of H$_V$ in phagocytes and other cells [10,11]. The VSP is a monomer whose VSD senses membrane potential and accordingly regulates the activity of the attached intracellular phosphatase [12]. (Adapted from [13].)

When a channel opens and conducts current, one result is a change in the membrane potential. The rule is that each channel drives the membrane potential toward its own Nernst potential. Thus, when Na$^+$ and Ca^{2+} channels open, they depolarize the membrane, whereas open K$^+$ channels tend to hyperpolarize the membrane. There are also a number of non-selective ion channels. When they open, they drive the membrane potential toward their Nernst potential, which is 0 mV; hence, they usually depolarize the membrane, because for the most part cells have negative resting membrane potentials. A cell with a large number of open non-selective ion channels bears a disturbing resemblance to an electrophysiologically dead cell, exhibiting a familiar *Gestalt* that is witnessed by electrophysiologists at the end of every experiment—a leaky membrane with no reaction to radical changes in bath solutions, and a relentless approach toward no membrane resistance and a 0 mV membrane potential. Even so, there are situations in which brief opening of non-selective ion channels serves a useful purpose. For example, non-selective endplate channels in skeletal muscle open in response to acetylcholine released from a motor neuron, depolarizing the muscle membrane enough to initiate an action potential that propagates along the membrane and produces contraction [16].

The *raison d'être* for proton channels differs in different species. A clue to their general function in multicellular species, in fact in all species except dinoflagellates [4], is that they are uniquely regulated by the pH gradient, ΔpH (defined as pH$_o$ − pH$_i$), in such a manner that they open *only* when there is an outward electrochemical gradient for H$^+$ (i.e. ΔpH > 0) [17]. As is discussed below in 'Why do proton channels have ΔpH-dependent gating?', when H$_V$ open, they always carry outward current. As a result, they

also tend to hyperpolarize the membrane. In certain situations, this effect provides identifiable benefit to the cell, but perhaps more frequently, the primary purpose appears to be regulating pH. When H$_V$ open, H$^+$ leave the cell. In other words, H$_V$ extrude acid, thereby increasing pH$_i$. One may speculate that H$_V$ exist in a large number of species because all cells engage in metabolism, which leads to a net production of protons that must be extruded to maintain a pH$_i$ compatible with cellular activities. Numerous other functions have been proposed that are specific to particular cells in particular situations [17]; full discussion of these is beyond the scope of this review.

Proton channels are extremely efficient proton extruders, capable of changing pH$_i$ an order of magnitude faster than other H$^+$ equivalent transporters [18–20], at rates up to 6 pH units min^{-1} in small cells [18], more slowly in large cells [21,22]. Because they do not require ATP [23], they can extrude acid at minimal metabolic cost to the cell. Under some circumstances, the concomitant extrusion of charge could incur some cost owing to the necessity of charge compensation. In one intensely studied case, however—the respiratory burst in phagocytes (figure 4)—the cell is already enthusiastically extruding negative charge in the form of superoxide anion produced by NADPH oxidase, so the activation of H$_V$ simultaneously extrudes acid and compensates charge. During the respiratory burst, proton channels set the membrane potential to precisely the value at which H$^+$ current is equal and opposite to the electron current [24]. The extraordinarily efficient activity of H$_V$ also includes two additional simultaneous functions. H$^+$ efflux compensates the massive electron flux without causing undue osmotic imbalance, because the biochemical products of H$^+$ inside the phagosome include a variety of membrane permeable
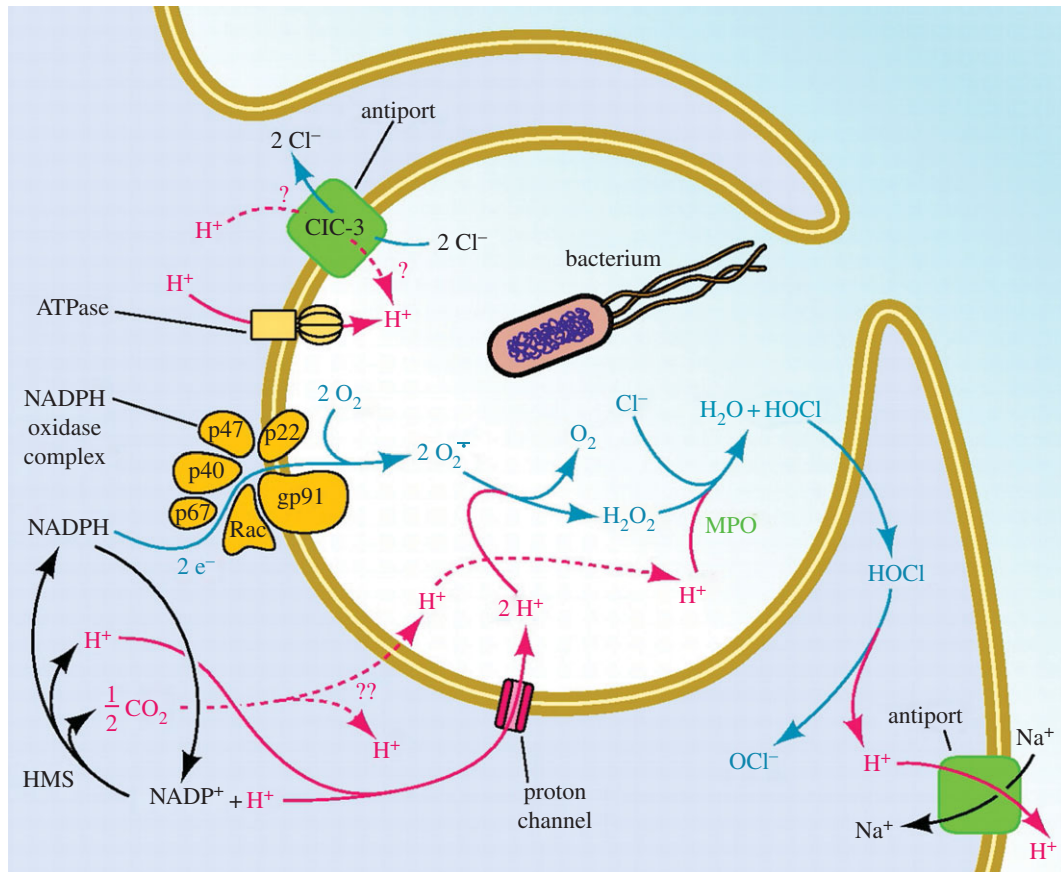
Figure 4. Proton pathways and proton transport proteins in phagocytes during the 'respiratory burst'. Part of a white blood cell (for example, a neutrophil) is depicted as its plasma membrane engulfs a bacterium. The enzyme NADPH oxidase (a.k.a. 'NOX2') assembles and becomes active in the membrane of the phagosome, the newly forming intracellular compartment enclosing the invader. NOX2 activity is so intense in neutrophils that it consumes orders-of-magnitude more oxygen as a substrate to form $O_2 \cdot^-$ than is used by the rest of cell metabolism combined, hence the misnomer 'respiratory burst' [24–26]. NOX2 is electrogenic, because it moves electrons from cytoplasmic NADPH across the membrane to reduce O_2 to $O_2 \cdot^-$ [27]. The prodigious electron flux permits NOX2 activity to be measured directly as electron current [28,29], which without compensation would depolarize the membrane by approximately 11 kV min^{-1} [23]. Voltage-gated proton channels compensate electrically for the electron flux, prevent large pH changes in cytoplasm or phagosome, minimize the osmotic consequences of charge compensation, and are a necessary substrate for producing H_2O_2 and HOCl [24]. (Adapted from [13].)

species (H_2O, H_2O_2 and HOCl) which can dissipate an osmotic gradient simply by diffusing through the membrane. Finally, H^+ flux into the phagosome is crucial because H^+ is required in large quantities as a substrate for the major reaction products, H_2O_2 and HOCl [24].

The teleological benefits of opening proton channels in different cells tend to fall into four general categories, each of which is exemplified in the phagocyte respiratory burst: (i) *electrical*—charge compensation, setting the membrane potential or generating action potentials, (ii) *control of pH_i* (intracellular pH), (iii) *control of pH_o* (extracellular pH) and (iv) *osmotic*—e.g. cell or organelle volume regulation.

(i) Charge compensation or regulating membrane potential by H_V has been proposed for neutrophils [27,30–35], eosinophils [32,36–41], monocytes [42], macrophages [20], osteoclasts [43], basophils [44], microglia [45,46], dendritic cells [47], sperm [48], B lymphocytes [49,50] and cardiac fibroblasts [51]. Producing more dramatic electrical effects than simply setting the resting membrane potential, proton channels in dinoflagellates exhibit properties consistent with the suggestion that they mediate the action potential that triggers a luciferase-induced bioluminescent flash [4,52–54].

(ii) Regulating pH_i is a general function, but has highly specific consequences in sperm capacitation [55], breast cancer cells [56], egg maturation [57,58], basophils during histamine release [44], neutrophils and eosinophils during phagocytosis [34,41,59–61] and coccolithophores during calcification [62].

(iii) Regulating pH_o is considered an important function of hH_V1 in human airway epithelium [63] and in exacerbating the invasiveness of breast cancer cells [64]. In the latter situation, H_V benefit the cells to the detriment of the organism.

(iv) Finally, osmotic effects are thought to be involved in H_V1 activity in microglia [65]. H^+ flux via H_V in phagosomes likely minimizes the volume changes that would occur if charge were compensated by other means [24].

In summary, the consequences of proton channel activity are highly diverse and depend strongly on the precise situation in each type of cell or organelle.

1.3. Why do proton channels need to be so selective?

Ion channels are often selective for one ion over others present in physiological solutions. That said, biology tends to be parsimonious—Na^+ and K^+ channels are selective, but

not extremely so, and in fact, they do not need to be. If the 'wrong' ion permeates 1% of the time, this will have a negligible impact on the cell. After all, Na^+ and K^+ gradients are being dissipated continually, particularly in excitable cells, and the Na^+/K^+-ATPase works continuously to restore these gradients. Na^+ channels still manage to mediate action potentials even though their error rate is substantial: P_K/P_{Na} is 0.05–0.1 [66–69] and as high as 0.23–0.30 when K^+ is inside and Na^+ outside [70], as usually is the case. Selectivity is often quantified as relative permeability (for example P_K/P_{Na} is the permeability to K^+ relative to that of Na^+), which can be calculated from measured reversal potentials (V_{rev}) using the Goldman–Hodgkin–Katz voltage equation [71–73]

$$V_{rev} = \frac{RT}{F} \log \frac{P_{Cl^-}[Cl^-]_i + P_{K^+}[K^+]_o + P_{Na^+}[Na^+]_o + P_{H^+}[H^+]_o}{P_{Cl^-}[Cl^-]_o + P_{K^+}[K^+]_i + P_{Na^+}[Na^+]_i + P_{H^+}[H^+]_i}.$$
(1.2)

Permeability means the facility with which a particular ion crosses the membrane. This will depend mainly on which ion transport proteins are present, because small ions cannot permeate biological membranes (lacking proteins) at physiologically relevant rates. If the membrane contains only one type of ion channel, permeability indicates how efficiently the ion permeates this type of channel. Note that permeability is a property of the ion and the channel in combination; increasing the concentration of ion X increases the conductance (g_X) calculated from the measured current (I_X) divided by the driving voltage ($V-E_X$), but does not change the permeability (P_X). The Goldman–Hodgkin–Katz equation (equation (1.2)) says that the ion with the highest permeability will dominate V_{rev}, at least when its concentration is reasonably high. In practice, measuring V_{rev} in solutions containing as few ion species as possible simplifies the calculation. K^+ channels tend to be more selective than Na^+ channels, with P_{Na}/P_K approximately 0.001–0.1 [73,74]. On the other hand, many K^+ channels are highly permeable to Tl^+ and Rb^+, and often P_{NH4}/P_K and P_{Cs}/P_K are a modest, but respectable 0.1–0.2. Ca^{2+} channels are more selective, with a relative permeability to physiological monovalent cations, P_K/P_{Ca} or P_{Na}/P_{Ca} of just 10^{-3} to 10^{-4} [73], although essentially all Ca^{2+} channels conduct certain other divalent cations (Sr^{2+} or Ba^{2+}) relatively indiscriminately [73] and often better than Ca^{2+}. Teleologically, Ca^{2+} channels need to be highly selective against monovalent cations because the Ca^{2+} concentration is substantially lower than that of K^+ or Na^+. All of these traditional ion channels can still do their jobs without being meticulously selective. Other channels are even less selective. Cl^- channels tend to be very poorly selective and conduct fairly large organic anions [75,76]; their virtue depends on the virtual absence of other anions in physiological solutions. Acetylcholine receptor channels at the neuromuscular junction are altogether non-selective among cations—their job is to depolarize the membrane just enough to initiate an action potential in the skeletal muscle membrane [16]. An open non-selective channel will drive the membrane potential toward 0 mV, and this rather imprecise effort is still entirely adequate to depolarize the membrane to the threshold ($V_{threshold}$) for opening voltage-gated Na^+ channels.

Proton channels, on the other hand, must be extraordinarily selective in order to accomplish anything useful.

The proton concentration is literally a million times smaller than that of other ions, so a truly impressive relative permeability of $P_H/P_K = 10^6$ would still allow similar numbers of K^+ (present inside cells at approx. 10^{-1} M) and H^+ (present inside cells at approx. 10^{-7} M) to permeate. In fact there is no evidence that any other ion permeates at all, which means H_V may be considered to be proton specific. Replacing the predominant cation or anion in the bath solution does not change V_{rev} detectably, once liquid junction potentials are corrected [23]. In some studies, changes in pH shift V_{rev} by an amount indistinguishable from the predicted shift in E_H [4,22,36,77–79]. More frequently, the measured V_{rev} deviates from E_H, but the explanation is simply that it is very difficult to control pH perfectly, especially when the measurement of V_{rev} itself requires activating large H^+ fluxes across the membrane. Reducing the ionic strength (decreasing the concentrations of all cations and anions, except for H^+ and OH^-) by 90%, a strategy intended to distinguish cation from anion permeable channels [80], does not change V_{rev}, independently confirming H^+ selectivity [7]. It is clear that proton channels both need and exhibit extreme selectivity. The question is—how do they accomplish this? One key to understanding proton selectivity lies in the mechanism by which protons move from point A to point B within proteins, and how this differs from the movement of other ions. This is discussed in the following section, before we return to the question of selectivity.

1.4. Proton transfer in liquid water and in proteins

The discussion below seeks to provide a qualitative description of the various chemistries used by nature to transfer protons within proteins. We purposefully exclude the important topics of the energetics and the mechanics of proton transfer. For entry to these topics, we suggest one of many reviews [81]. We first consider proton transfer in liquid (bulk) water, then proton transfer through a single file of waters and finally the roles of protein side chains.

The key to all of the proton transfer events discussed here is movement of the proton through a well-aligned hydrogen bond [82–84]. It seems a loosely kept secret that hydrogen bonds abound in liquid water, therefore the mechanism of proton transfer should be simple (!). The ion at the centre of proton transfer through water is hydronium (H_3O^+), with the slight complication that hydronium never exists as a free ion in bulk water, only as an idealized structure [85]. Most of the time, protons in liquid water are contained within Eigen cations ($H_9O_4^+$), composed of a hydronium ion with hydrogen bonds to a primary hydration sphere of three waters [86,87]. At any instant, the hydronium is closer to one of its primary sphere waters than it is to the other two; the hydronium and its closest water are designated the 'special pair' [88]. A new special pair forms, on average, every 40 fs. After several picoseconds, one of these special pairs becomes a Zundel cation ($H_5O_2^+$; $H_2O-H^+\cdots OH_2$) and achieves the optimum hydrogen bond geometry that allows the proton to be shared between two waters ($H_2O-H^+-OH_2$), and then transferred to the acceptor water ($H_2O\cdots H^+-OH_2$) [88]. This event is often termed 'proton hopping' because the proton has, in essence, hopped from a lone pair of electrons on one oxygen to a lone pair on the acceptor oxygen. Following the proton transfer event in the Zundel cation, the Eigen cation re-forms. A recent study
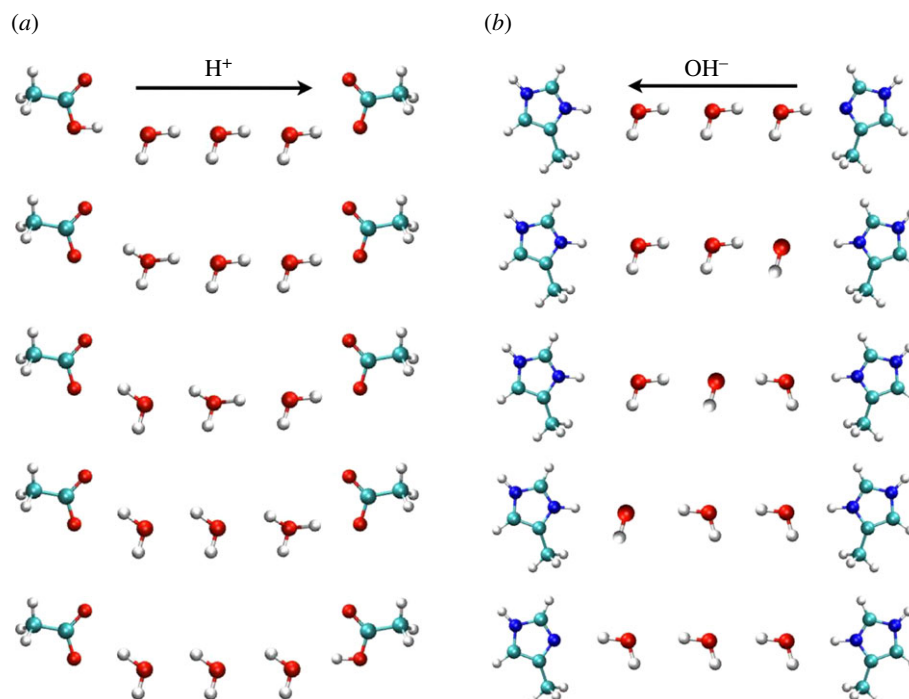
Figure 5. Conduction of H^+ (a) and OH^- (b) occurs by protons hopping (a) by the classical Grotthuss mechanism as an excess proton hopping from donor H_3O^+ to acceptor H_2O, or (b) as proton 'hole' migration, respectively. In the latter case, the net leftward migration of OH^- results from a series of retrograde hops of H^+ to the right. The net result in both cases is the transfer of one proton from left to right, and these processes are indistinguishable electrophysiologically. Reprinted with permission from [96]. Copyright © 2006 American Chemical Society.

produced a somewhat different picture: the proton moves rapidly (less than picosecond) along a short wire of two to three hydrogen-bonded waters, but rests as a Zundel cation for longer periods (2–5 ps) between these concerted hops [89].

The through-bond character of proton hopping enables protons to effectively diffuse through water rather than around it, as other ions must. In 1936, Huggins [90] noted that 'the charge shifts (through hydrogen-bonded waters) with little actual motion of the atoms'. In fact, the mobility of H^+ through water is 4.5–7 times greater than that of other common physiological ions (K^+, Na^+, Ca^{2+} or Cl^-) [91]. In 1905, Danneel [92] explained the anomalously high mobility of protons in water as the Grotthuss mechanism, in recognition of work a century earlier by Freiherr von Grotthuss [93,94]. Grotthuss-type proton transfer in water also appears to be a component of Brønsted acid–base chemistry. Acids dissolved in water, for example acetic acid, transfer their proton to a base through a hydronium intermediate (actually the hydronium within the Eigen cation) [95].

Now we turn our attention to proton transfer within proteins, using lessons learned from liquid water as well as studies of H^+ channels, H^+ pumps and enzymes that include H^+ pathways. A proton transfer pathway or proton transfer chain (PTC) is a sequential series of proton carriers that follow a structurally defined route within a protein. In enzymes, a PTC leads from the proton source (e.g. bulk water) to the proton sink (e.g. the active site or a redox centre). The reverse direction applies when protons are released by the chemistry within the protein. In transmembrane proton pumps, one PTC leads from solvent to the internal proton-pumping element, while a second PTC leads from the pumping element to solvent on the other side of the membrane. The proton carriers of a PTC are either side chains of amino acids or internal water molecules; in most known PTCs within proteins both of these components are involved. As in liquid water, a key

requirement is hydrogen bonding. Each component of a PTC must be capable of hydrogen bonding both the preceding and the next proton carrier, although this may not occur simultaneously, as discussed below.

A PTC composed solely of a single file of waters, a water wire, is perhaps the simplest pathway to visualize (figure 5a). A proton enters a water file by forming a covalent bond with the first water to form a hydronium ion. The hydronium ion may hydrogen bond to the next water to form the Zundel cation, $H_5O_2^+$ [97]. In the confined channels of PTCs in proteins, formation of the yet larger Eigen cation is precluded by geometrical constraints when the channel diameter does not exceed 4 Å [98]. The mechanism of proton transfer in a water wire is likely to be modified from that of bulk water because the waters of the linear water file can form a maximum of three hydrogen bonds, one fewer than waters in bulk [99]. Nevertheless, successive proton hops between waters in the file can rapidly move the proton from the source to the sink. Several of the waters may align their hydrogen bonds for concerted hopping, and the proton may rapidly hop back and forth along these groups of waters prior to reaching the proton sink [99,100]. In order to move a second proton from source to sink, it is necessary for each of the waters to turn 180°, thus presenting an open lone pair on each water O to the incoming proton [101,102]. The overall speed of proton transfer through a transmembrane water file is rapid, and can occur in less than 500 ps in gramicidin channels, because single-channel currents corresponding to more than 2×10^9 H^+ s^{-1} have been reported [103]. This is a record for a selective ion channel, eclipsing the BK Ca^{2+}-activated K^+ channel, which conducts a mere 10^9 K^+ s^{-1} at +300 mV in 3.4 M K^+ [104]. When a single file of waters is confined in a hydrophobic channel with a diameter of 4.0 Å, the calculated proton transfer rate is several-fold faster than in bulk water [98]. Evidently,

even at atomic dimensions, laminar flow is more efficient than turbulent flow. In a linear water chain, the proton can hop forward or backward along the file, but not sideways or up and down. Moreover, a protein may construct a channel that optimizes the positions of the waters for rapid proton transfer [99]; branching in a hydrogen-bonded water network slows proton conduction [105].

The quintessential example of a purely aqueous proton pathway through a protein spanning the membrane is the gramicidin channel. This short peptide (a dimer of 15-amino acid monomers) produced by *Bacillus brevis* forms a cylindrical tube filled with water [106]. The interior is hydrophilic; peptide bond carbonyl groups coordinate a single-file row of a dozen water molecules [107]. Titratable groups, for example carboxyls, are absent. Gramicidin is permeable to a variety of monovalent cations, but it excels at conducting protons [108]. Normalized to the permeant ion concentration, the proton conductance of gramicidin is 100-fold greater than that of other ions [109]. The facility of proton permeation arises from their ability to hop along the water file without displacing the waters, whereas other cations must wait for the water molecules to diffuse through the pore ahead of them [109]. As mentioned above, H^+ flux at rates exceeding $2 \times 10^9 \, s^{-1}$ have been recorded in gramicidin channels [110]. The example of gramicidin shows clearly that a narrow pore filled with water is capable of conducting protons with high efficiency, but the transfer is not selective, because other cations pass, albeit more slowly.

1.4.1. A digression on why proton channels carry such tiny currents

The human proton channel hH_V1, has an estimated conductance of 78 fS at physiological pH and human body temperature [111]. Given a large driving force, say 100 mV, one channel would carry 7.8 fA of H^+ current, which translates into $5 \times 10^4 \, H^+ \, s^{-1}$. Does this mean that H_V are not efficient proton channels, in comparison with gramicidin, for example? Perish the thought! The gramicidin value mentioned above $(2 \times 10^9 \, H^+ \, s^{-1})$ was obtained at $+160$ mV in 5 M HCl! Our hH_V1 can withstand a pH range beyond anything they encounter in the human body, but at pH < 4.5 or pH > 8.5 they, or the membranes they reside in, do not survive long enough to permit study. By contrast, when incorporated into synthetic lipid bilayers, gramicidin continues to function at pH -1, in 10 M HCl [110]! It is revealing to note that the single-channel proton current through gramicidin is almost directly proportional to H^+ concentration over a wide pH range from 0 to 4.5 [23,110,112]. Extrapolated to the pH range where H_V1 single-channel measurements are possible (pH 5.0–6.5) [111], the gramicidin H^+ current is actually smaller [23]. We conclude that H_V are very respectable proton channels that carry small currents simply because the concentration of their permeant ion (H^+) is extremely low in physiological solutions.

1.4.2. Involvement of amino acid side chains in proton transfer within proteins

Long water wires do exist within functional PTCs in proteins. The 'D' pathway of the mitochondrial enzyme cytochrome *c* oxidase (C*c*O) is proposed to be a file of 8–10 hydrogen-bonded waters that transfers protons from a surface-exposed aspartic acid residue to an internal glutamate residue [113,114]. One fascinating aspect of the D pathway is that the continuity of the water file is constantly being broken and restored at one point by an amide side chain that moves in and out of the water file [115]. Nevertheless, proton transfer through the waters remains rapid [115,116]. The functional pathway for proton transfer to the quinone-binding site of *Escherichia coli* succinate dehydrogenase may involve up to 13 waters [117].

With the possible exception of the succinate dehydrogenase pathway [117], native proton transfer pathways in proton pumps and enzymes involve side chains of certain amino acids (particularly carboxylates) along with internal waters. The chemistries of the various side chains increase the number of mechanisms available for proton transfer. For example, in the 1970s, Nagle and others [101,102,118] recognized that certain side chains of amino acids, for example hydroxyls, could participate in proton transfer without ionization, i.e. without the net dissociation of a proton. In such a mechanism, the hydroxyl group uses hydrogen bonds to simultaneously acquire a proton from an upstream proton carrier and release its proton to a downstream proton carrier. Chemically, a lone pair of electrons on the hydroxyl O acquires a H^+ while another pair releases a H^+. Throughout the process the hydroxyl remains as $-OH$, avoiding ionization to either $-O^-$ or $-OH_2^+$. In theory, a long chain of hydrogen-bonded hydroxyl groups could carry out concerted proton transfer over a considerable distance. This is not seen in known or proposed PTCs in proteins, perhaps because the precise alignment of hydrogen bonds is difficult to sustain over a long series of hydroxyl groups. In fact, when the hydroxyl groups of serine, threonine and tyrosine do appear in the proton transfer pathways of proteins, they do so individually, and they are bracketed by carriers capable of forming a stable ion, such as a carboxyl or water [23]. For example, a recently identified proton pathway in *Clostridium pasteurianum* [FeFe]-hydrogenase comprises a five-member motif with Ser bracketed by two Glu residues (Glu[282], Ser[319], Glu[279], H_2O, Cys[299]) [119].

The acidic and basic side chains of amino acids add conventional acid–base chemistry to the realm of proton transfer. Most significantly, the fact that proton acquisition and proton release are *separable* events for an acid or base brings the aspect of rapid side chain movements into play. For example, at the entrance to the D pathway for proton transfer of C*c*O, a carboxylate side chain accepts a proton from solvent (water), then the protonated carboxyl group rotates 180° about the terminal C–C bond of the side chain, establishes a hydrogen bond to the oxygen of the first water of the D pathway and transfers the proton to this water [120]. Just the rotation of the protonated carboxyl moves the proton approximately 2.2 Å along the trajectory of the transfer path. (The approximate centre-to-centre distance of the O atoms of a carboxyl group is 2.2 Å; a proton cannot hop from O to O of the carboxyl because the geometry of the atoms precludes an intramolecular hydrogen bond.) In C*c*O, the rotation of this D pathway carboxyl moves the proton from 'outside' to 'inside' [120]. The crystal structures of C*c*O show no space for hydronium or other ions to move around the carboxyl, so its rotation constitutes a *proton-specific gate*. The chemistry of such carboxyl rotation has been studied in detail in a bacterial ferredoxin, where a critical aspartic acid residue also moves a proton from outside to inside [121]. Examination of other protein structures suggests that carboxyl rotation may be common in proton transfer pathways.

The imidazole group of histidine is analogous to a carboxyl, in that it contains two atoms (N_δ and N_ϵ) that can be protonated, but a proton cannot hop between these two sites because there is no intramolecular hydrogen bond. Similarly to carboxyls, neutral imidazole can be protonated on the open N, rotate 180°, and then deprotonate to the next proton carrier [122–125]. In this case, the rotation moves the proton approximately 2.1 Å, the centre-to-centre distance of the N atoms of the imidazole ring.

Transfer of protons can occur via larger, but still rapid, movements of whole protein side chains (rather than just carboxyl or imidazole ends of side chains), e.g. the swinging motion of a lysine within the K pathway of C*c*O [126,127].

1.5. How can one design a proton selective channel?

With the basics of proton transfer in mind, let us take up the question how a proton channel may select for protons. A proton channel requires two features to be selective for protons. First, there must be a proton transfer pathway, using any of the options described above for the transfer of protons through proteins. As we have seen, the mechanisms of proton transfer in proteins are closely related to proton acid–base chemistry in solution, and therefore a PTC in a channel protein is inherently selective for protons. Based on PTCs in other proteins, we might expect the PTCs of ion channels to include both waters and amino acid side chains. Although the channel must supply a pathway for the proton that spans the entire membrane, the proton *selective* section of the pathway may be shorter. In principle, selectivity can be achieved by directing all of the protons to pass through a single titratable side chain, such as a carboxyl or an imidazole group, at some point in the channel. As is the case with many ion channels, H_V1 has large aqueous vestibules (figure 1), separated by a narrow region approximately 10–12 Å long in which water is mainly single file [3] (figure 1). This short, narrow region is the section of the transmembrane proton pathway that is thought to confer selectivity for protons.

Second, proton selectivity requires some mechanism to prevent other ions from also flowing through that region of the channel that contains the PTC. *A priori*, it would seem that a straightforward design strategy would be close protein packing. The passage of monovalent cations, for example, requires an open space, a pore, through which the ions can move. By contrast, proton transfer requires *no* open space, because the H^+ may be transferred as part *of* the protein. As it moves from carrier to carrier through the protein via H-bonds, the H^+ is covalently associated with lone pairs of electrons on internal waters or protein side chains. Given this fundamental difference in the structural requirements for proton transfer versus the transfer of other ions, it would seem that evolution could produce a highly selective proton channel simply by filling in the space around the PTC component of the channel.

1.5.1. Of water wires and Na^+

Water wires (water files) have been proposed for part or all of the PTC component of some H^+ channels. The H^+ selectivity of these channels varies. At this time, H_V1 is the only one that appears to be completely proton selective (Table 1 of [17]). The influenza virus M2 channel is highly proton selective, but also conducts K^+ and Na^+ [128–130]. A synthetic hydrophobic α-helix composed of leucine and serine (i.e. lacking formal charges) oligomerizes to form a channel containing a water wire that conducts H^+ more than 40-fold better than Li^+, based on the presence of detectable single-channel currents [131].

There is no doubt that a water file forms an excellent pathway for protons; the question is how to enforce proton selectivity. A relevant paradigm is the transmembrane H^+ or Na^+ channel of the F_o–F_1-type ATP synthases. In some bacterial versions of these enzymes, the transmembrane channel of the F_o rotor transfers Na^+, but in related enzymes (including mitochondrial ATP synthase), the channel is specific for H^+ [132,133]. The transmembrane channel of F_o is constructed of two water-filled half channels leading to and from a controlling group located in the middle of the membrane [134,135]. In the Na^+ channels, the central group is a multi-valent coordination site for Na^+, while in the H^+ channels the central group appears to be a single carboxyl side chain [136]. For the H^+-conducting ATP synthases of *Spirulina platensis* and *Bacillus pseudofirmus*, spectacularly selective binding (10^8–10^9 higher for H^+ than Na^+) is demonstrated by the F_o rotor being driven by H^+ at pH 9 and 200 mM Na^+ [137,138]. This does not prove that Na^+ cannot reach the input half-channel; simply that it does not bind and catalyze enzyme activity. However, the working enzyme translocates H^+ across the entire membrane with superb selectively. Hence, nature appears to have conferred H^+ selectivity upon the transmembrane channel of many F-type ATP synthases by replacing a Na^+-binding site with a single carboxyl side chain. The elucidation of further structural factors that confer H^+ selectivity in the F-type ATP synthases should be highly instructive for other H^+ channels, and vice versa.

Other more subtle and difficult to validate mechanisms have been proposed for H^+ selectivity by water wires. One such is the 'frozen water' hypothesis, some form of which has been proposed for the M2 viral proton channel (figure 6*b*) discussed below [140–142], for the synthetic proton channel of Lear *et al.* mentioned above [131,143], and for H_V1 [144,145]. In this mechanism, one or more waters at a narrow region in an aqueous pore are constrained by the pore walls, essentially frozen in place in such a way that they can still translocate a proton by the Grotthuss mechanism, while simultaneously preventing other cations from permeating. Although nominally frozen, the waters need to retain enough mobility that they can reorient after each proton conduction event [101,102].

Another way to enforce proton selectivity still envisions a water wire, but one that is continuous only transiently. Molecular dynamics simulations of hH_V1 in the open state indicate that a continuous water wire exists just 10% of the time (figure 1), and it persists only for a nanosecond or so [3]. One could speculate that protons, speedy little devils that they are, could zip through such a pathway, whereas much bulkier and slower ordinary cations could not permeate within such a short-time window. Such a situation is proposed for the normal function of the D pathway of C*c*O, although not as a solution to the problem of ion selectivity. Although the D pathway transfers protons at rates at least up to $10^4 s^{-1}$ [116], high-resolution structures show a clear discontinuity in the water file of this pathway in the form of two amide side chains of Asn^{139} and Asn^{121} [127,146]. Molecular dynamics simulations suggest that the amide side chain of Asn^{139} spontaneously swings out of the way to allow formation of a continuous water file [115]. The rate
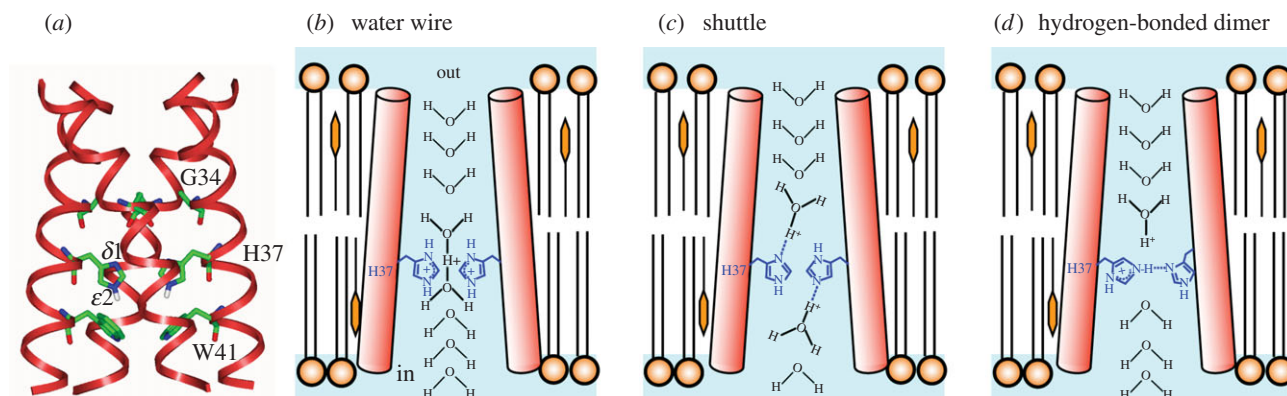
Figure 6. The well-studied M2 proton channel of influenza A virus is a homotetramer; (*a*) shows the high-resolution crystal structure with key residues labelled. Three proposals for the proton selective conduction are illustrated in (*b–d*), with only two of the four protomers shown for clarity. In the water wire model (*b*), the proton pathway is exclusively aqueous, with protonation of multiple His[37] serving to open the pore by electrostatic repulsion. Proton selectivity in the shuttle model (*c*) is achieved by successive protonation and deprotonation of His[37], with a ring flip completing each conduction event. In (*d*) protonated and unprotonated His[37] form hydrogen-bonded dimers that are broken during H$^+$ conduction. (Adapted from [139].)

of H$^+$ transfer remains rapid because the movements of protein side chains and internal waters are even more rapid.

Yet another hypothetical mechanism for proton selectivity postulates a continuous water wire that is energetically or electrostatically unfavourable to ions. We have seen how a proton can move back and forth along a file of waters. This reduces the effective charge of the proton at any specific location, i.e. the charge is delocalized [85,97,99,147,148]. By contrast, the charge on an ion, for example Na$^+$, exists at a discrete location; so it becomes possible for a protein structure to purposefully exclude such a charge. A delocalized-charge mechanism has been proposed for a synthetic proton channel [149].

1.5.2. Aquaporin: a water pathway that excludes protons

A paradigmatic example of a water-filled channel that excludes protons is aquaporin (AQP), a membrane protein that provides a pathway for rapid water permeation across the membrane [150], but excludes all ions including protons [151,152]. The structure of AQP revealed a narrow region 20 Å long at the centre of which is a pair of NPA (Asn–Pro–Ala) motifs from nearby short helices [153]. It was proposed that the two Asn amido groups formed hydrogen bonds with a single water molecule, preventing the water from hydrogen bonding with adjacent waters, thereby precluding proton conduction [153]. Shortly after this, a nearby 'constriction region' with conserved Arg, His and Phe residues was identified [154], later called the 'selectivity filter'. A number of molecular dynamics studies ensued to evaluate the structural and physical basis for blockage. Initial simulations in the absence of H$^+$ were interpreted to support the water orientation hypothesis [155], but subsequent calculations showed that the free energy barrier opposing the movement of an excess proton through the pore peaks at the NPA motif [156–159]. Several of the latter studies concluded that ion blockage results from electrostatic effects combining the ion's desolvation penalty and interactions with charged and polar groups of the channel. A simpler model was proposed by Burykin & Warshel [160,161], who argued that AQP with all of the ionizable groups in the channel neutralized, and even a 4 Å hole in the membrane exhibited a large energy barrier to proton permeation, and concluded that the desolvation penalty for protons to leave bulk water and cross the centre of the membrane was itself

sufficient to exclude protons. However, point mutations of the selectivity filter region, R195 V or R195 V/H180A, were identified that enabled proton conduction through AQP1 [162,163]! These results showed that neutralizing the charge of R195 in the selectivity filter suffices to confer proton permeability, despite the continued presence of the NPA region and the desolvation penalty for inserting protons into the narrow pore.

AQP can be mutated to conduct protons, whereas H$_V$1 can be mutated to exclude protons. In the human H$_V$1 channel (figure 1), the Asp^{112} crucial to proton selectivity is salt-bridged with Arg^{208} about 90% of the time, so their charges are neutralized. When Asp^{112} is neutralized by mutation (e.g. to Ser or Ala) molecular dynamics simulations reveal that the charge owing to the now-unpaired Arg^{208} produces a 10 kcal mol^{-1} barrier to cations [3]. It was shown recently that Asp^{112} can be shifted one turn of the helix outward to position 116 without loss of proton selectivity [164]. Molecular dynamics simulations reveal that at its new location, Asp^{116} is generally paired with Arg^{208} or Arg^{205}. There is an electrostatic barrier to cation permeation when Arg^{208} is unpaired, but not when it interacts with Asp^{185} or Asp^{116} [164]. Evidently, proton exclusion can be achieved by an unpaired cationic group in a critical location. It is well established that the charge selectivity of anion-selective, ligand-gated channels can be switched to cation selectivity by introducing negatively charged Glu at key locations [165,166], and that cation selective channels can be made to conduct anions by various mutations that include neutralization of Glu [167,168]. These rules of charge selectivity can be used to destroy proton selectivity, but producing proton selectivity is a subtler task.

1.6. The extensively studied M2 viral proton channel as a model

The M2 channel of the influenza A virus [169] is a favourite example of a highly proton selective ion channel [170–172]. This channel is relatively simple, being a homotetramer of 97-amino acid monomers (figure 6). Four transmembrane helices of M2 proteins associate to form a single channel that transfers protons into an endosome containing a virus that has been taken up by a cell. Proton uptake initiates the loss of

the viral protein coat. The M2 channel is not as selective for protons as the H_V1 channel, and was shown recently to exhibit measurable permeability to Na^+ and K^+ [128–130,173], which tarnishes it somewhat as a paradigm of perfect proton permeability. Even so, the M2 proton channel selects for H^+ over Na^+ or K^+ by a factor of approximately 10^6 or more [128–130,170,173]. As discussed below, this is achieved by proton transfer through a His^{37} cluster, combined with physical occlusion of Na^+ or K^+ transfer through the His^{37} cluster. The rate of H^+ transfer from exterior to interior is relatively slow at $100\,s^{-1}$ or less by most accounts [128–130,171,173–176]; a gate composed of a ring of Trp^{41} side chains [177,178] slows reverse proton transfer even more.

From the analysis of site-directed mutants and high-resolution structures, the key to H^+ selective conductance is His^{37} [179,180]. As the channel assembles as a tetramer of four identical α helices, there is a tetrad of His^{37} whose side chains face the centre of the pore [181]. His^{37} plays key roles both in activating the channel and in proton selectivity. Protonation of two His^{37} opens the channel [141,176,182,183]. H^+ conduction is mediated by the third His^{37} to be protonated [139,169,184], and its relatively high pK_a may limit H^+ flux [139,185]. Mutagenic alteration of His^{37} (for example H37G) abolishes proton selectivity [179]. The mechanism by which His^{37} imparts proton selectivity (figure 6c) was originally suggested to be protonation of the imidazole δ-nitrogen from the extracellular side of the membrane, deprotonation of the imidazole ε-nitrogen to the intracellular side, followed by a ring flip to restore the orientation of the singly protonated state [186]. This shuttle mechanism can be described quantitatively by simple mathematical models [184,187,188]. Because each H^+ conduction event entails conformational changes of His^{37} and Trp^{41}, one could reasonably define M2 as a carrier rather than a channel. The essentials of this model remain, i.e. protonation and de-protonation of His^{37} plus a flipping motion of the imidazole ring. Further details of the mechanism are currently debated, as discussed below.

High-resolution crystal structures (figure 6a) and NMR structures have provided considerable structural information about the rings of His^{37} and Trp^{41} residues and the positions of internal waters [169,176]. In addition, the existence of hydrogen bonds and the protonation state of the His^{37} can be determined using NMR spectroscopy [139,189–191]. These data allow for detailed models of proton transfer through His^{37} [169,176,192]. In two current models, the selectivity filter for protons involves just three components and two proton transfer steps: a hydronium from the exterior aqueous region of the channel transfers a proton to the His^{37} cluster (which is organized differently in each model), and then the His^{37} cluster transfers the proton to a water below the ring of Trp^{41} side chains, when this Trp^{41} gate is open to allow a hydrogen bond to form between a component of the His^{37} cluster and the acceptor water molecule.

In the mechanism proposed by Zhou, Cross and co-workers [176,183,192], based primarily on calculations on an NMR structure and further spectroscopy, the deprotonated His^{37} tetrad is organized as two hydrogen-bonded imidazole–imidazolium dimers (figure 6d). The introduction of a proton from an upstream hydronium breaks the H-bond of one of these dimers (either one, apparently) producing two imidazolium side chains that rotate and swing away from each other. One swings down to transfer a proton to a downstream water revealed by the opening of the Trp^{41} gate. The

de-protonation of this His^{37} allows re-formation of an imidazole–imidazolium dimer.

The mechanism (figure 6c) of Hong, DeGrado, and co-workers [169,193] is primarily based upon a 1.65 Å crystal structure of the transmembrane channel [194] combined with solid-state NMR dynamics data. In this structure, the orientation of the side chains of the ring of four His^{37} roughly resembles that seen in the NMR structure used by Zhou, Cross, *et al.* but the side chains are evenly spaced around the ring and direct H-bonds between side chains are absent. Rather, the four imidazole/imidazoliums form a hydrogen-bonded network with associated waters, which are located atop the His^{37} ring and between the histidines and the Trp^{41} ring [194]. The histidine side chains and the waters are capable of extensive delocalization of an added proton; continuous ring flips of the histidines facilitate the sharing of the added proton with the waters between the His^{37} and Trp^{41} rings. The three components of the PTC in the Hong–DeGrado model are a hydronium from the aqueous channel above the His^{37}/water cluster, the His^{37}/water cluster itself and a water to receive the proton below the Trp^{41} gate. The rate-limiting step in H^+ conduction is imidazolium ring reorientation [169].

A principal difference in the two models is the nature of the His^{37} structure that is protonated by a single proton from the exterior side of the channel and then de-protonated by water beyond the Trp^{41} gate. In the Zhou–Cross model, the His^{37} proton transfer group cycles between an imidazole–imidazolium dimer and two imidazolium side chains, while in the Hong–DeGrado model, the His^{37} structure is a His^{37}/water cluster in both its protonated and de-protonated forms, whose degree of hydration increases as the degree of protonation is increased. Evidence that the hydrogen-bonding partner of His^{37} is water instead of another His^{37} was obtained from ^1H NMR chemical shifts [193]. A swinging movement of the histidine side chains appears less required in the Hong–DeGrado model, because either of the waters located between His^{37} and Trp^{41} in the His^{37}/water cluster can mediate proton transfer to the water beyond Trp^{41}.

1.7. What makes hH$_V$1 selective?

Returning to our main interest, the voltage-gated proton channel, our current explanation of proton selectivity consists mainly of describing which parts of the molecule are involved. Because no crystal structure exists for the channel in any species, what we believe about structure is derived from homology modelling based on crystal structures of voltage-gated Na^+ and K^+ channels (specifically, their homologous VSDs) and on molecular dynamics simulations in conjunction with experimental data. As illustrated in the snapshot in figure 1, hH$_V$1 narrows to a region approximately 10 Å long containing more or less a single file of waters that most of the time is interrupted at an Asp^{112}–Arg^{208} salt bridge [3]. Experimentally, the PTC within H_V appears to consist of more than water. For example, a number of characteristics of proton permeation through H_V led to the idea that the rate-limiting step occurred inside the pore, not in the approach through bulk solution [195–198]. Postulating a PTC including one or more titratable groups [18,197,198] could explain why the high selectivity, deuterium isotope effect and temperature dependence of conduction differed drastically from proton conduction through the water-filled gramicidin channel [109,199,200], but were

similar to the corresponding properties of the M2 channel [170,171, 201,202], in which proton conduction entails protonation/deprotonation of His residues [186].

The H^+ selectivity of voltage-gated proton channels in both humans [7] and dinoflagellates [4] requires an aspartate residue in the middle of the S1 transmembrane segment, at a narrow region of the pore (figure 1), Asp^{112} and Asp^{51}, respectively. Certainly, H^+ selectivity could be achieved by a mechanism in which Asp^{112} is protonated and deprotonated by every proton transferred. When Asp^{112} is replaced with a neutral amino acid, H_V not only lose their proton specificity but become preferentially permeable to anions. Phenomenologically speaking, that this *Gestalt* is identical in both species indicates that the selectivity mechanism is strongly conserved evolutionarily, given that there is only 14.6% identity between the two proteins [4,17]. Replacing Asp with Glu preserved proton specificity, but other mutants, including the His mutant, D112H or D51H, respectively, were permeable to anions [4,7]. Recent demonstration that the selectivity of hH_V1 is preserved when Asp is moved from position 112 to 116, one turn of the S1 helix outwards (but not to a dozen other positions tested) reiterates the role of Asp, but indicates some structural latitude in the placement of the H^+ selective carboxyl group [164].

There exists a clear preference for carboxyl side chains (Glu or Asp) in proton transfer pathways of pumps and enzymes [120,121,203–220], often as the group that introduces protons into the pathway. The D pathway of CcO has been established as an experimental system for examining the ability of side chains to sustain steady-state proton transfer [120]. In the normal D pathway, the initial proton acceptor is the carboxyl group of a conserved aspartic acid. Histidine cannot replace Asp as the initial proton acceptor at the normal position [221]. The thiol of Cys can function in this system as an initial proton acceptor, but only when it is moved to a point in the pathway with significant structural constraints that limit rotamer possibilities [120,221]. The preference for a carboxyl group for proton uptake is likely due to several factors: its structure is analogous to two waters connected by a carbon, its functional pK_a is readily modified by the surrounding protein, and it has the capability to rapidly rotate end-over-end—like a turnstile—through a compact protein structure in order to transfer a proton to the next element in the PTC [120].

Nevertheless, simply locating an Asp in a pore-lining position is not enough to produce proton selectivity. In the *c* subunit of H-ATPase, the key carboxyl of Asp^{61} can be moved to a different helix (D61G/A24D), but not to nearby positions 58, 60 or 62 on the same helix [203,222]. Asp^{185} also faces the pore in hH_V1 [3,144,145,223] but can be neutralized by mutation (D185S, D185N, D185V) without affecting proton selectivity [7]; conversely, Asp^{185} is still present in D112x mutants (i.e. mutants in which Asp^{112} is changed to His, Lys, Asn, Ser, Ala or Phe) that lack H^+ selectivity [7]. As shown in figure 1, the pore is likely wider at the level of Asp^{185} [3]; evidently Asp must reside at a narrow point to produce selectivity. A compact protein structure around the carboxyl will prevent the passage of other ions.

That the His mutant of hH_V1, D112H, was permeable to anions [7] was initially surprising, in view of the strong similarity of the transmembrane domain of H_V1 to the VSD of K^+ and Na^+ channels [1,2]. The VSD includes the first four transmembrane helices (S1–S4), which are thought to act as the voltage-sensing component of the channel; they mechanically couple to the pore domain, which comprises the four S5–S6 segments (figure 3). Although the VSD of K^+ or Na^+ channels does not normally conduct current, when the outermost of a series of Arg residues in the S4 helix is replaced by His, a voltage-gated proton conductance appears [224–226]. This result supports the view that the VSD is roughly hourglass-shaped with aqueous vestibules that access both sides of the membrane and focus onto a short occluded region [224]. When His is located at this constriction, it selectively translocates protons [224–228]. Why does His introduced at a comparable region in hH_V1 not shuttle protons? A crucial difference is that in the mutant K^+ channel VSD, His replaces a cationic Arg, whereas in the D112H mutant of hH_V1, His replaces a negatively changed Asp. In hH_V1, Asp^{112} normally apposes the cationic Arg^{208} (figure 1); mutation of Asp results in uncompensated positive charge that excludes protons and produces anion selectivity [3]. Intriguingly, in aquaporin, proton exclusion occurs at the 'ar/R region', in which Arg^{195} apposes His^{180} [163].

In ATP synthase, the crucial acidic group Asp^{61} (in *E. coli*) exists in proximity to a highly conserved Arg^{210} residue, called the 'stator charge' whose function is thought to be to ensure release of the proton from the acidic binding site [133,136,203,229,230]. In an intriguing parallel, the proton selectivity element in hH_V1, both at its native position, Asp^{112}, and when repositioned to Asp^{116}, is found by MD simulations to be mainly engaged in salt linkages with one or more Arg residues in the S4 helix [3,164].

1.8. What is the difference between H^+ and OH^- channels?

It turns out that distinguishing H^+ and OH^- channels is very difficult. The electrochemical gradient that drives conduction of either species is always identical. For example, at pH_o 7.5, pH_i 6.0, the Nernst potentials are $E_H = E_{OH} = -87$ mV (at 20°C). At symmetrical pH, applying $+100$ mV to the inside of a cell (or an isolated membrane) drives H^+ out and/or OH^- in, resulting in currents that are indistinguishable to a voltage-clamp circuit. The effects on pH on either side of the membrane are also identical; moving H^+ out increases pH_i and decreases pH_o by the same amount as moving OH^- in. It is likely that most conduction of H^+ and OH^- occurs by closely related mechanisms involving proton hopping (figure 5) by a Grotthuss-like mechanism [89,92–94,231–234]. In the chemical systems in figure 5, H^+ is more likely to carry the current when donor and acceptor are acidic ($pK_a < 7$), whereas OH^- conduction (as a proton 'hole') is more likely when they are basic (pH > 7) [96].

As discussed above, the human voltage-gated proton channel, hH_V1, is proton selective because of a specially located aspartic acid residue (Asp^{112}) [7], which is almost certainly negatively charged *in situ*, with a low pK_a. When this Asp^{112} is mutated to a neutral amino acid (e.g. Ser, Ala and Asn), the channel becomes anion selective. This charge reversal is understandable if we consider that, in all likelihood, the negatively charged Asp was compensated in the WT channel by one or more nearby cationic charges, so that elimination of the Asp leaves unpaired cationic charges. However, although Cl^- is clearly permeant in D112x mutants, the preferred charge carrier appears to be OH^- by several orders of magnitude (in terms of relative permeability, P_{OH}/P_{Cl}, although not necessarily in real numbers) [7]. Why this should be is not obvious. The pathway in WT hH_V1, comprising an aqueous
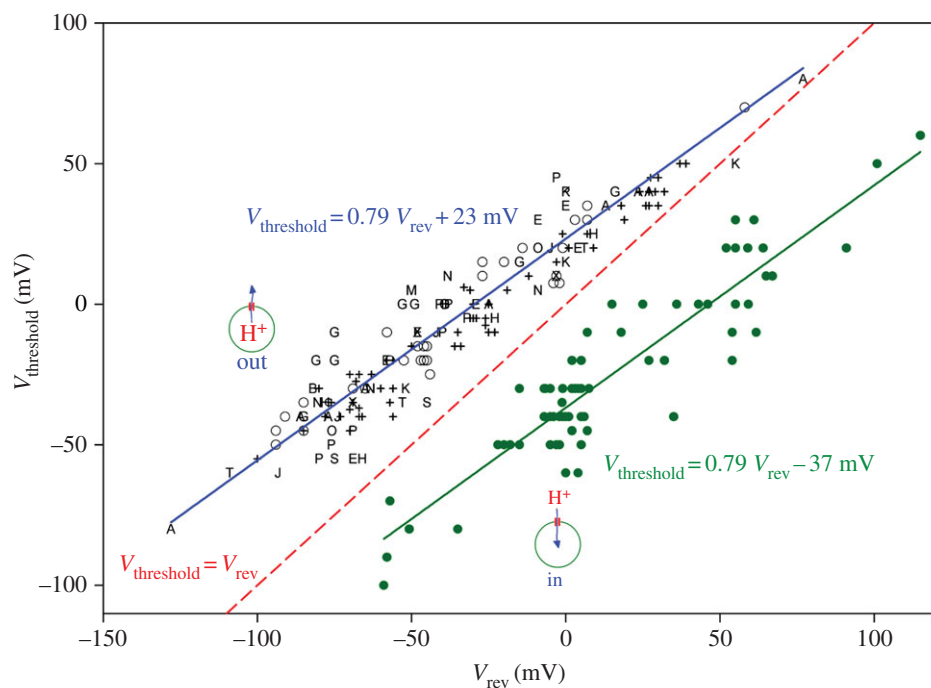
Figure 7. The property of ΔpH-dependent gating is universally conserved in proton channels, but exists in two forms. In most cells (15 varieties are illustrated here as various symbols), proton channels open only when there is an outward electrochemical gradient, and this results in acid leaving the cell. This pattern is seen over a very wide range of pH_i and pH_o; $V_{threshold}$ is always above the dashed line of equality. In the single case of the dinoflagellate *K. veneficum* (filled circles), $V_{threshold}$ for the kH_V1 channel varies with identical slope with changes in pH_i and pH_o; but at any given ΔpH, $V_{threshold}$ is 60 mV more negative than in other H_V1; this means that opening kH_V1 produces inward proton current under all conditions. Most cells use H_V1 to eliminate excess acid, but dinoflagellates have other goals in mind, such as conducting action potentials and triggering a bioluminescent flash. (Adapted from [17].)

pore that most of the time is interrupted at an Asp^{112}–Arg^{208} salt bridge [3], supports a high rate of H^+ flux. Mutation of Asp^{112} alters the electrostatic environment drastically, by replacing a net-neutral salt bridge with an uncompensated cationic charge, producing a 10 kcal $mole^{-1}$ barrier for cations [3]. Facile OH^- permeation should not be too surprising, because the mechanism of OH^- conduction also involves H^+ hopping and occurs rapidly. Because OH^- permeates by means of proton hopping (figure 5b), it has tremendous mobility advantages over Cl^-, both because of its small effective size and the rapidity of proton hopping (discussed above).

In several tissues that exhibit H^+/OH^- flux at extremely high pH, such as *Chara australis* at pH 10.5 [235,236] or frog skeletal muscle at pH 10 [237], OH^- flux seems more likely than H^+ flux simply because there are vastly more OH^- than H^+ to act as charge carriers.

In summary, the consequences of H^+ or OH^- permeation are similar, the mechanism of transport is similar, and which species is transported may depend on the pK_a of critical groups in the pathway or on the net charge at a narrow part of the channel.

1.9. Why do proton channels have ΔpH-dependent gating?

A great many ion channels are voltage gated, which means that they have a high probability of being open (and conducting their particular favourite ion) at certain membrane voltages and a high probability of being closed (not conducting anything) at other voltages. Most voltage-gated ion channels, including our favourite, H_V, open when the cell membrane is depolarized. For most ion channels, the voltage dependence is absolute, subject to modulation by permeant ion effects discussed below. A K^+ channel that opens at −40 mV in

physiological solutions will carry outward current. The same channel exposed to elevated external K^+ concentration will still open at −40 mV but will carry inward current. Channel opening depends only on voltage; once a channel is open, the direction of current flow depends only on the electrochemical driving force ($V − E_{ion}$). There are rather subtle effects of permeant ion species and concentration on the gating of several channels [238–240], most famously the 'occupancy hypothesis' of Clay Armstrong in which occupancy of the pore by a permeating ion slows the closing of the channel, also called the 'foot-in-the-door' effect [241]. $ClC−0$ Cl^- channels [242,243] and inwardly rectifying K^+ channels [244,245] have more pronounced permeant ion effects on their gating than most other channels. In contrast with H_V, however, these effects are exerted by permeant ions mainly from just one side of the membrane.

One of the most extraordinary properties of every voltage-gated proton channel identified to date is ΔpH-dependent gating [17,18,23,246,247]. The effect of proton concentration (pH) on the gating of proton channels is not only pronounced, but it occurs equally on both sides of the membrane [248]. The consequence is that the position of the proton conductance–voltage (g_H–V) relationship is determined by the pH gradient, ΔpH, defined as $pH_o − pH_i$ [248].

Figure 7 illustrates the practical consequences of this unique ΔpH dependence. Here, we indicate the position of the g_H–V relationship in a convenient format in terms of $V_{threshold}$, the 'threshold' voltage at which proton channels first start to open. Data collected from studies of 15 different cell types are all described well by the upper line. $V_{threshold}$ is plotted as a function of the reversal potential, V_{rev}. One could equally replace the experimentally determined V_{rev} with the more theoretical value of the proton Nernst potential, E_H, because the perfect selectivity of H_V means that V_{rev} should be very close to E_H. In practice, however, it is not so easy to establish pH accurately, even when

one uses such high buffer concentrations that buffer is practically the only anion in the solution. The proton channel is a superb pH meter that reports precisely the pH gradient across the membrane. For this reason, we prefer to believe what H_V tells us about pH, which is contained in V_{rev}. Returning to figure 7, we see that $V_{threshold}$ is always positive to V_{rev}—the dashed line shows the point at which they are equal. Finally, the punch line: proton channels do not open unless the electrochemical gradient is outwards, as it is for all the symbols on the graph (except the solid circles). Therefore, when the proton channel first opens, it will conduct acid out of the cell. *Voila!* The primary function of proton channels is acid extrusion!

Why do cells need to extrude acid? (Here, we will be kingdomcentric and consider mainly animal cells.) As was cogently stated by Roos & Boron [249] in their historic 1981 review, 'the central problem of pH_i regulation [is] the neutralization of intracellular acid derived from a variety of sources' [249, p. 365]. Cells produce acid continuously during metabolism and are constantly looking for ways to get rid of it. From this perspective, the worst possible thing is a pathway that would allow H^+ to leak into cells! The ΔpH dependence of proton channels ensures that this disaster will never happen.

Nevertheless, for the proton channel to be most useful, it does need to open reasonably near E_H so that as soon as enough acid builds up, proton channels will open and release it. A quibbler might argue that the ΔpH dependence illustrated in figure 7 results in $V_{threshold}$ changing only 40 mV unit^{-1} change in ΔpH, insufficient to parallel E_H which changes 58 mV unit^{-1}. This point is technically valid, but arguably may not reflect a design flaw. Perhaps the approach of the upper solid and dashed lines in figure 7 means that $V_{threshold}$ is closest to E_H when there is a large outward gradient, i.e. when the cell has become very acidic and is in dire need of relief from acid build-up.

A persistent quibbler might point out that $V_{threshold}$ is an artificial concept, because the probability that proton channels open (P_{open}) does not have a true threshold, but decreases exponentially with hyperpolarization. From this perspective, a larger cushion between $V_{threshold}$ and E_H might be a prudent strategy in the negative voltage range, where cells may sit at their resting potential for days on end, and even a tiny H^+ influx might have untoward cumulative consequences.

Fine, but what about the solid dots in figure 7? In the phylogenetic tree in figure 2, we saw that the proton channel that differs most in amino acid sequence from all the others is kH_V1, a dinoflagellate proton channel. This sequence difference is reflected in a crucial functional difference, which is obvious from the dots in figure 7. Although kH_V1 has ΔpH-dependent gating with precisely the same slope as all other proton channels, the absolute position is offset by -60 mV [4]. The consequence of this unique set-point is that when kH_V1 first opens, it conducts inward current. The biological effects of channel opening could not be more different! Acid enters the cell instead of leaving. Why would this ever be desirable?

One answer is that this property would allow the proton channel to mediate an action potential. Conducting an action potential requires a conductance that activates negative to its Nernst potential [250,251]. As is discussed above, two familiar examples are voltage-gated Na^+ and Ca^{2+} channels, both of which activate negative to their Nernst potentials and do in fact conduct action potentials [252–254]. The proton channel identified recently in a non-bioluminescent dinoflagellate species, *K. veneficum*, was found to activate well negative to E_H over a wide range of pH (figure 7), precisely the behaviour needed to

mediate an action potential [4]. These properties strongly support suspicions that proton channels mediate the action potential that triggers the bioluminescent flash [4,23,53,255].

As it turns out, several bioluminescent dinoflagellates, when stimulated mechanically by waves or other disturbances of the seawater, emit a rapid flash that can be very impressive when plankton blooms concentrate many individuals in one location [256]. Forty years ago, voltage-gated proton channels were first conceived by J. Woodland Hastings, who proposed that they trigger the flash [52]. This response occurs when a stimulus evokes an action potential in the large intracellular flotation vacuole [257–260]. The action potential invades the scintillons [261,262], numerous evaginations of membrane that form small vesicles [263] that are packed with luciferin, luciferin-binding protein and luciferase [255,264–266]. Because the vacuole has very low pH of 3.5–4.5 [54,267], an open proton channel will conduct protons very rapidly into the scintillon, where the pH is near neutral [268]. Luciferase is quiescent at high pH, but is activated by a drop in pH to approximately 6.5 [255]. Thus, the influx of protons through H_V rapidly lowers the pH of the scintillon and thereby activates luciferase. An additional parallel mechanism is that luciferin-binding protein releases luciferin (the substrate for luciferase) at low pH [255,265,269].

In order for H_V to carry out their function, they must open negative to E_H and conduct inward current. Figure 7 shows that this is precisely what they do. Any other proton channel would just sit there, refusing to conduct inward current, because the vacuole interior is the topological equivalent of extracellular space. The discovery of the unique properties of kH_V1 [4] confirms Woody Hastings's hypothesis, nearly 40 years later. It turns out that having a $V_{threshold}$ negative to E_H is required not only to mediate the action potential, but also to activate luciferase in the scintillons!

1.10. Why do proton channels in mammals exist as dimers?

In most 'higher' species, H_V1 exist as dimers [270], as illustrated in figure 3, with the peculiar property that monomeric constructs exhibit all of the main physiological features of the dimer [8,9,223,271,272]. This situation raises the question, why bother to dimerize? Proteins assemble into multimers for a variety of reasons. Heteromultimers obviously need to assemble all their parts to make a complete, functioning complex. Some membrane proteins are more stably expressed when they form multimers; as monomers they are rapidly degraded. For example, two key membrane-bound components of the NADPH oxidase complex, $gp91^{phox}$ and $p22^{phox}$ reside in the membrane as a heterodimer, but do not remain in the phagocyte membrane when mutation prevents one from being expressed [273]. However, monomeric H_V constructs appear content to function indefinitely in cell membranes.

The most consistent functional difference between monomeric and dimeric H_V is in gating kinetics. Monomeric constructs open approximately five times faster than dimeric channels [8,223,271,274,275], as is evident by comparison of figure 8a,b. This distinct difference in speed might seem to be important in some situations, until we remember that among species, activation kinetics varies over several orders of magnitude. H_V in snail neurons open within a few milliseconds [246], whereas mammalian H_V require seconds or even minutes [31]. It would appear that a species with a need for rapidly opening proton channels would be better served by
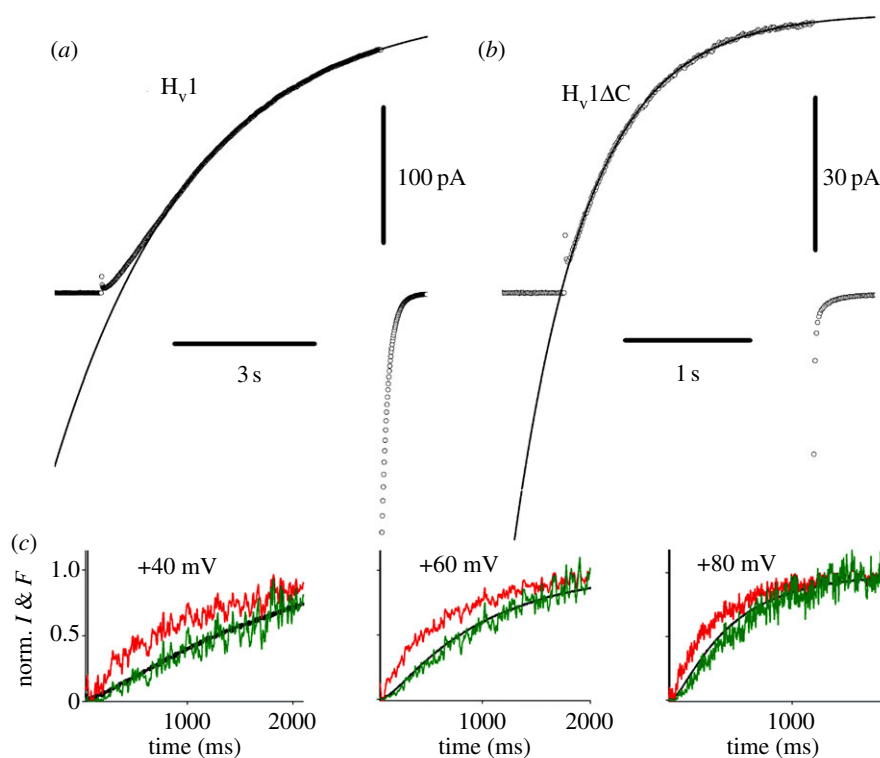
Figure 8. Kinetics of proton currents generated by the normal dimeric human proton channel, hH$_V$1 (a) and a monomeric construct produced when the C terminus was truncated, hH$_V$1ΔC (b). The dimeric channel, which occurs in native cells [276], opens with sigmoid kinetics and approximately five times slower than monomeric constructs (note different time scales). Conditions are the same, pH$_i$ = pH$_o$ = 7.5, 23°C, voltage pulses to +50 mV. In (c), the S4 helix of the *Ciona intestinalis* proton channel was labelled with a fluorescent tag that moves when the channel opens upon depolarization (upper noisy traces), preceding the activation of current (black line). In a Hodgkin–Huxley type cooperative gating mechanism [252], both subunits move before either conducts and the predicted time course is sigmoidal, the square of the motion time course (lower noisy traces, superimposed on line) (a,b adapted from [223]; c from [272]).

making a channel that is intrinsically fast, rather than tinkering with stoichiometry. This perspective reveals no obvious advantage of dimeric over monomeric architecture.

A more subtle property of dimeric H$_V$1 channels is that they gate cooperatively [272,275]. Gonzalez *et al.* [272] attached a fluorescent tag on the S4 transmembrane helix, which could be seen to move upon depolarization with an exponential time course (upper noisy traces in figure 8c) that preceded the turn-on of H$^+$ current (smooth lines in figure 8c). The implication is that both monomers must respond to voltage before either one can conduct. In such a system, the effective gating charge that moves when the channel opens is doubled. Opening a tetrameric K$^+$ channel requires movement of all four of its VSDs, and the charge moved is quadruple that of a single VSD. The upshot is that by requiring both protomers to move before either can conduct, H$_V$1 has twice as steep a dependence on membrane potential [223,272,277].

What good is this? Consider the plight of a neutrophil that has just engulfed a bacterium (figure 4). In order to produce sufficient ROS to kill the microbe, thousands of NADPH oxidase complexes quickly assemble in the phagosome membrane, and begin to transfer electrons from NADPH in the cytoplasm across the membrane to reduce O$_2$ to O$_2 \cdot ^-$. The separation of charge, electrons moving across the membrane and protons left behind, causes rapid depolarization. In fact, if this process were not compensated, the membrane potential of a human eosinophil would depolarize at a rate of approximately 18 V s^{-1} [23], and membrane dielectric breakdown would occur in a tiny fraction of a second. Clearly, something must be done.

To limit the depolarization, cations must leave or anions must enter. As we saw in figure 4, phagocytes have determined

that the best solution to this problem is to open proton channels and extrude H$^+$ to balance the transmembrane electron transfer. It turns out that NADPH oxidase is sensitive to membrane potential; it is inhibited by depolarization, and turns off altogether at about +200 mV [32]. Of course, this problem is self-imposed—it is the electrogenic activity of NADPH oxidase [27] that causes the depolarization. Fortunately, depolarization opens proton channels, and the greater the depolarization, the more channels open (and the greater the driving force for H$^+$ efflux), until the electron efflux is perfectly balanced by proton efflux. In this remarkable system, NADPH oxidase activity causes depolarization and lowers pH$_i$ and both of these factors open proton channels, which in turn have the effects of limiting the depolarization as well as minimizing changes in pH$_i$. Despite the efficiency of H$_V$, the membrane potential still depolarizes enough, roughly to +50 mV in human neutrophils [278–280], to impair NADPH oxidase activity by approximately 15–20% [24]. Matters would be worse if hH$_V$1 were not dimeric, however, because the weaker sensitivity to membrane potential of a monomeric hH$_V$1 would mean that greater depolarization would be needed to open enough proton channels, and consequently, more self-inhibition of NADPH oxidase activity would ensue [223]. That hH$_V$1 is dimeric makes NADPH oxidase more efficient.

Although this explanation applies specifically to H$_V$1 in phagocytes, it could also apply in a more general way for other mammalian cells. Steeper voltage dependence means that a smaller depolarization is required to open enough H$^+$ channels to do any job. This makes H$_V$1 more efficient, and less of a drain on the resources of the cell.

Finally, there are several unicellular species in which H$_V$ are probably monomers [4,270], because the protein lacks the

coiled-coil domain in the C terminus that is the main enforcer of dimerization [8,9,223,271,272,281,282]. The original question was 'Why are some H_V dimers?' We will ignore the obverse question 'Why are some H_V monomers?' because peer pressure seems inadequate as a basis for evolution.

1.11. Concluding thoughts

The field of voltage-gated proton channels is expanding rapidly in all directions: its presence in new species, its presence in new cell types, novel functions, involvement in normal physiology as well as in disease, and advances in structure–function relationships. The function of H_V in all situations depends on its selectivity for protons as well as a unique ΔpH-dependent gating mechanism. Both properties are subjects of intense study. The mechanism of proton selectivity involves an aspartate in the centre of the pore; further details are unclear. The mechanism of ΔpH-dependent gating is remains obscure. The proton channel field has benefited from work on other proton-conducting molecules and on VSDs of other voltage-gated ion channels. As our understanding of H_V develops, one hopes that proton channels will return the favour.

Endnotes

[1]The total includes 10 papers that are only partly about proton channels but excludes seven papers whose authors thought they were studying proton channels, but in all probability were not.

[2]Since the dawn of electrophysiology over two centuries ago in the heyday of Luigi Galvani, cells have been known to have an electrical potential across their plasma membranes. During an action potential, or nerve impulse, this 'polarization' was reduced or eliminated, a process described as 'depolarization' (literally removal of polarization), which was thought to reflect a transient breakdown of the membrane potential. Similarly, 'hyperpolarization' described an increase in polarization beyond the normal resting membrane potential. Once it became clear that the membrane potential transcended 0 mV during the action potential [14,15], it was necessary to redefine the terminology. Today depolarization simply means making the membrane potential more positive, and hyperpolarization means making it more negative.

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