

Review

Et tu, Grotthuss! and other unfinished stories

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Abstract

This review article is divided into three sections. In Section 1, a short biographical note on Freiherr von Grotthuss is followed by a detailed summary of the main findings and ideas present in his 1806 paper. Attempts to place Grotthuss contribution in the context of the science done at his time were also made. In Section 2, the modern version of the Grotthuss mechanism is reviewed. The classical Grotthuss model has been recently questioned and new mechanisms and ideas regarding proton transfer are briefly discussed. The last section discusses the significance of a classical Grotthuss mechanism for proton transfer in water chains inside protein cavities. This has been an interesting new twist in the ongoing history of the Grotthuss mechanism. A summary and discussion of what was learned from probably the simplest currently available experimental models of proton transfer in water wires in semi-synthetic ion channels are critically presented. This review ends discussing some of the questions that need to be addressed in the near future.

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1. Et tu, Grotthuss!

2006 marks the bicentennial anniversary of the famous Grotthuss publication on the effects of the electric field on decomposition of water and solutes [1]. The 1806 paper is a reproduction of a French pamphlet that was published in Rome in December 1805. Even though I am not aware of publications referring to the Grotthuss mechanism for proton transfer (discussed in detail in Fig. 1 in the next section) in the XIX century, similar models became popular since the beginning of the XX century. It is likely that Grotthuss paper is more quoted than read. It was a fascinating experience to read the original 1806 paper, to contrast Grotthuss research with other science done at that time, and to relate his guesses to today's views of ion and proton mobility in water.

Perhaps surprisingly, several leads about Grotthuss were found in the Internet. Starting at the most popular Grotthuss website (the four-star Grotthuss Hotel in Vilnius, Lithuania), the website from the "Lithuanian Royal Union of Nobility" [2] was soon found. In that site, a short review by Grotthuss biographer

Krikštopaitis is found (website addresses are listed as references at the end of this article). Freiherr Christian J. Theodor von Grotthuss was born in Leipzig in 1785. He was the son of an aristocratic German family that moved from Westphalia to the Baltic region by the end of the XVIII century. Grotthuss resided in Paris during 1803–1805 and there he "became a pupil of French science". In Paris, Grotthuss attended lectures of famous French scientists and became acquainted with Volta's pile. It was Gay-Lussac who introduced Grotthuss to experimental science [3].

In 1805, Grotthuss and Gay-Lussac went to Mount Vesuvius to investigate the volcanic eruption that took place earlier that year. It was in Naples and Rome (1805–1806) that Grotthuss did the experimental work that resulted in his famous publication. In 1807, Grotthuss went back to Vilnius. He suffered from severe depression and pain of an "inherited disease" of the pancreas (personal communication from Dr. Krikštopaitis). Incapable of doing science and of visiting major scientific centers, Grotthuss reasoned his life meaningless and committed suicide in 1822 (Krikštopaitis, personal communication). Portraits of Freiherr Grotthuss and references to published biographies are also found in some websites [2].

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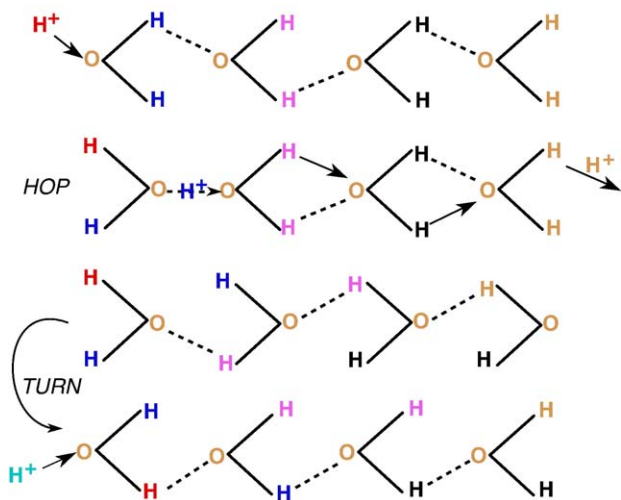


Fig. 1. A highly schematic representation of a unidimensional chain of four water molecules interconnected via H-bonds (water or proton wire). The explanation for the high mobility of protons in bulk water is detailed in the text and has been the classical textbook explanation since the beginning of the XX century. This has been thoroughly questioned recently (see text).

Grotthuss paper is divided in two chapters. In this and in the next paragraph, italics are texts either in French or in English translations of some passages. The first chapter of the article had fifteen numbered paragraphs. In that chapter, Grotthuss described in detail the effects of *galvanic electricity* (the potential difference between a zinc and copper electrodes immersed in the same salt solution) on particles (*corps*) dissolved in water. Between the zinc and copper electrodes immersed in the same solution (lead acetate or tin chloride in water), Grotthuss described the most *beautiful images of shrubs with their own foliage* extending from the negative (copper) to the positive (zinc) electrode in solution. Those images were compared to *plants searching for sunlight and growing in that direction*. Those processes did not touch the electrodes but ended close to them. However, not all salts provided those beautiful images. With iron salts for example, Grotthuss described a gradual change in the color of the solution between the electrodes. That gradation could be reversed by simply inverting the positions of electrodes in solution.

Chapter II (paragraphs XV through XXV) develops a theory of water decomposition caused by *galvanic electricity*. *In analogy with the positive and negative poles of a voltaic battery that immortalized the genius of his creator Volta*, and in an *inspirational burst (trait de lumière)* Grotthuss reasoned that the water molecule must also possess negative and positive poles. *Due to a simple contact or friction between their bodies* (hydrogen *h* and oxygen *o*) *natural electricity* is partitioned in a water molecule in such a way that *h* and *o* acquire a *positive and a negative state*, respectively. As such, one of the electrodes immersed in the solution attracts oxygen and repels hydrogen while at the other electrode, the opposite happens. This is the basic phenomena underlying water decomposition: when a water molecule (*o h*) gives away its *o* at the positive pole its remaining *h* is immediately re-oxygenated by the arrival of an adjacent *o* whose *h* then recombines with another *o*, etc.. A

similar phenomenon would happen in the opposite direction with the *h* at the negative pole. In this case the moving particle is the *h* (today this is known as proton hopping). These two phenomena would occur until all water molecules are decomposed into *o* and *h*. It must be noticed that the H₂O nature of water was not established until 1811 by Avogadro, and that in Grotthuss explanation *both oxygen and proton hop* to a dissociated water (*o* or *h*) molecule. Intriguingly, a mechanism similar to the hopping of *o* and *h* was not discussed in regard to the electrochemistry of salts which were studied in Chapter Premier in the paper.

From Grotthuss writings, it appears that the idea of a chain of aligned molecules transferring a *body* was suggested by images of shrubs with their own foliage between electrodes. These shrubs between the electrodes contain paths in which bodies are transferred along. Faraday's lines of electrical forces seem to have been inspired by Grotthuss electrochemical experiments [3,4].

The idea that water is decomposed into two charged particles of opposite polarities that move along chains of water molecules until they form hydrogen and oxygen gases at electrodes was established in analogy with charge separation in a voltaic battery. This idea was brilliant, and it may well be the first time that a molecule (using modern terminology) is reasoned as comprised of particles of distinct electrical polarities!

Batteries were always present in well-equipped laboratories at that time [5]. In the early years of the XIX century, Humphry Davy isolated Na, K, and Ca using the largest battery in the world available at that time at the Royal Institution in England [6,7]. Davy's science was more quantitatively oriented than Grotthuss's. Because of his electrochemical work, in 1808 Davy traveled to France in the middle of a war between these countries to receive a prize awarded by Napoleon. Parenthetically, Davy protested against *the small-minded people who objected to his travel merely because England was at war with France* [5]. During that time it was also known that electricity generated either by a battery or by frictional machines was of the same kind. Notice that Grotthuss used the term *friction* between *h* and *o* to explain the development of their electrical polarities inside the water particle.

By the turn of the XVIII century, Laplace was already a big name in science. His attention by then was directed to capillarity, surface tension, and elasticity. These phenomena relate to intermolecular interactions, and the prevailing view was that "cohesive forces were probably gravitational in origin and so followed the inverse-square law at large distances but departed from that law at short distances where the shapes of the particles affected the interaction" [8]. Newtonian mechanics was still the inspiration for understanding physicochemical phenomena, and this was not going to change dramatically for another 30–40 years. The idea of attractive (or repulsive) electrical forces was not present in discussions of capillarity, chemical affinities and mutual attraction of the particles of the bodies [8]. Grotthuss guess of the water particle being comprised of positively and negatively charged bodies could have provided an initial inspiration or suggestion for reasoning intermolecular interactions as weak electrostatic phenomena

between molecules. Have electrical forces between bodies or molecules been considered by Laplace and others and not given further attention? Grotthuss work was certainly known by Faraday [4] and even later on by Planck [9] who unsurprisingly considered his ideas too mechanical. Thus, it is unclear why intermolecular interactions were not thought as electrical interactions.

Perhaps, the most interesting idea in Grotthuss paper is that water is comprised of one negative and one positive corpuscle (*o* and *h*, respectively). These corpuscles can dissociate and diffuse under an electric field. As properly acknowledged by Grotthuss, this idea was established in analogy with the positive and negative poles in a voltaic battery. The 1806 paper was revived 100 years later in a completely different context by Danneel [10] to explain the “abnormally” high mobility of H^+ and OH^- in water. At this time, the mobility of salts in solution was already reasoned in hydrodynamic terms. Of particular relevance to the current Grotthuss mechanism (see Fig. 1) is that an *h* is transferred between an *o h* molecule and the dissociated *o* along a chain of water molecules. While the notion of consecutive transfers of *h* between an (*o h*) and *o* molecules along a chain of molecules is certainly present in today’s Grotthuss mechanism, other essential ingredients were missing in the original formulation. Danneel [10] recognized [11,12] the need of rotation (the turn step in a modern Grotthuss mechanism) of a water molecule in sequential transfers of protons in chains of waters. The modern classical Grotthuss mechanism has attributed the rate limiting step in proton mobility in bulk water to the sequence of reorientations of water molecules (see below for a recent discussion of this problem). Another essential concept in a modern Grotthuss mechanism is the presence of H-bonds [13] between waters [11,12]. In fact, structural reorganizations of H-bonds are now believed to underlie proton transfer.

In balance, Grotthuss paper has been celebrated in relation to his work and ideas regarding the electrochemical decomposition of liquid water. In the beginning of the XX century his mechanism, now understood as involving proton hopping, has been rediscovered as a consequence of the failure of classical hydrodynamic models to explain the high mobility of protons in water. In closing this section, it should be noticed once more that Grotthuss also proposed the hopping of oxygen corpuscles, and he did not discuss the mobility or separation of metals in water.

2. Grotthuss rediscovered: the mobility of protons in bulk water in the XX and early XXI centuries

In the first decades of the XX century, the mobilities of several ions at infinite dilution were known. Ionic mobilities were reasoned as the resultant of two forces, (i) the applied electric field favoring, and (ii) the solution’s viscosity dragging the solvated ion ([14] and references therein). Intermolecular interactions were not considered. Overall, the predictive power of this theory is not good, and at most approximate. However, the largest errors occurred in predicting the mobilities of H^+ in water (assuming diffusion

of H_3O^+ , the calculated mobility was ~ 6.5 -fold smaller than measured) and OH^- (~ 3.3 -fold smaller). The equivalent H^+ mobility in dilute HCl solutions is $\sim 3.6 \times 10^{-3} \text{ cm}^2/(\text{s} \times V \times M)$. By contrast, K^+ has an equivalent mobility of $\sim 0.8 \times 10^{-3} \text{ cm}^2/(\text{s} \times V \times M)$. Because isolated protons do not exist in aqueous solution [76], the diameter of the smallest possible protonated water cluster (H_3O^+) is $\sim 3 \text{ \AA}$. This is comparable to a hydrated K^+ (3.3 \AA). Thus, H^+ mobility cannot be explained by hydrodynamic diffusion. Proton mobility was considered ‘anomalous’, and proton hopping as in the original Grotthuss mechanism (section 1 above) was revived [10,11,14,15].

Fig. 1 illustrates the basic ideas of a modern version of the Grotthuss’s mechanism. Consider four water molecules interconnected via H-bonds (water or proton wire, [16]). In Fig. 1, there is an electrochemical gradient favoring the movement of H^+ from left to right. The approach of H^+ (first row in Fig. 1) to the O of the first water molecule in the chain will eventually lead to formation of a covalent OH bond. One of the protons that was originally covalently linked to the O of water 1 will now be shared between waters 1 and 2 forming a protonated water dimer (Zundel’s cation, $(H_5O_2)^+$, 2nd row in Fig. 1). This hopping step propagates along the water wire (2nd row in Fig. 1). As the H^+ hops, the dipole moment of the water molecule donating the H^+ reverses. Once the H^+ leaves the last water molecule in the water chain of Fig. 1 (2nd row), the total dipole moment of the chain is reversed (3rd row in Fig. 1). If another H^+ is to be transferred in the same direction as before, the four water molecules need to rotate back (turn step) to their configurations (4th row, Fig. 1). In other words, a ‘red’ H^+ enters and a ‘green’ H^+ leaves the water wire (as the hopping step described by Grotthuss himself).

Until the mid 1990s the prevailing idea was that the rate limiting step of H^+ mobility in water was the rotation of water molecules as illustrated in Fig. 1 [10,11,14,15]. Significant discrepancies between the activation energies for water rotation and H^+ mobility at various temperatures were noted [17]. At temperatures higher than $\sim 293 \text{ K}$ there is good agreement between those activation energies. As the temperature decreases, however, differences between those energies become increasingly larger. At relatively low temperatures, water molecule can be coordinated via H-bonds with up to four other water molecules. For a water molecule to rotate at a low temperature, four H-bonds in the first solvation shell of (H_3O^+) must be simultaneously broken. The activation energy of this process exceeds that of H^+ mobility. At larger temperatures, water molecules lose H-bonds [18], decreasing the activation energy of water rotation and making it approach the activation energy for H^+ mobility.

What is the rate limiting step of H^+ mobility in bulk water? One idea is that the rate limiting step for H^+ transfer between two adjacent waters depends on the dynamics of water molecules in the second solvation shell of a Zundel cation ($H_5O_2^+$) [17,19,20]. The disruption of one H-bond in the second (or first) solvation shell of an H_3O^+ (or $H_5O_2^+$) creates the electrostatic conditions for the H^+ to hop between the adjacent oxygens in a Zundel cation. The

energy of this H-bond (~ 2.5 kcal/mol, [18]) is about the same as the activation energy of H^+ mobility in bulk water. In bulk water, it takes approximately 1 ps for a H^+ to cover a distance of 2.5 \AA [17].

Calculation of activation energy and H^+ mobility in liquid water at those short distances are in agreement with measurements [21]. However, a more “realistic” picture of H^+ transfer in bulk water is missing. In addition to refining the molecular dynamics methodology, it would be of interest to perform calculations with large water clusters that can accommodate sequential H^+ transfers between water molecules. How a water cluster that donates a proton “reprimed” itself for accepting another H^+ has not been addressed and is not clear. It seems to be also necessary to add an electric field or an electrochemical gradient during molecular dynamics (MD) simulations. These would mimic the experimental conditions used to measure H^+ mobility in water and H^+ transfer in biological molecules (see below), and would certainly revise the random walk picture of proton transfer presently studied. These gradients may unravel distinctive features of solvated proton clusters like weakening of some H-bonds and a preferred (re)orientation of water molecules. Another parameter that has not yet been correctly calculated by MD is the kinetic isotope effect (KIE) for H^+ mobility in water (~ 1.4). This is a complex issue [22,23]. It seems that a proper reaction coordinate that describes proton transfer (including the solvation shells) has not yet been properly defined and this is critical for the simulations of the isotope effects [24].

Whatever the mechanism by which H^+ are transferred between water molecules, it seems clear that an appropriate geometric arrangement between waters and H^+ is necessary for proton hopping. Consequently, it is not difficult to predict qualitatively that the conductivity or mobility of H^+ should decrease as the concentration of HCl in water increases or the structure of solution changes [25,26]. Any ion or molecule that intercalates itself between protonated water clusters would have the effect of blocking H^+ transfer between waters. For example, at high pressures or HCl concentrations the mobility of H^+ is similar to Cl^- [27–30], and H^+ mobility should be determined by the hydrodynamic properties of solvated protons [31]. In general, the mobility of H^+ should be determined by a combination of (i) a Grotthuss-type mechanism and (ii) the hydrodynamic diffusion of protonated water clusters [25,31].

3. Another twist in the history of the Grotthuss mechanism: H^+ transfer in water wires inside biological molecules

The presence of an approximately unidimensional chain of water molecules in cavities of various proteins adds another interesting twist to the history of the Grotthuss mechanism. After all, it is possible that the hop-turn steps discussed in Fig. 1 apply to a very special case of H^+ transfer in protein waters. It is possible that the rate limiting step could be the reorientation of water molecules [32]. However, this is not clear even in simple water wires (section 4 below).

H^+ transfer in water wires occurs in various bioenergetic proteins. The structural substrate for this transfer is summarized below:

(A) A chain of 14 water molecules extending over $\sim 25 \text{ \AA}$ from the protein interior to the cytoplasmic side is present in the photosynthetic reaction center of *Rhodobacter sphaeroides*. High resolution structures of the reaction center suggest that a network of H-bonded water wires together with polar side chains of amino acids underlie the transfer of H^+ from the cytoplasm to the quinone Q_B buried inside the protein [33–35];

(B) In cytochrome *c* oxidases, two distinct H^+ conduction pathways were identified (K and D channels). The K channel extends from the bulk aqueous phase on the electronegative side (bacterial cytoplasm) to the heme-copper center of the protein. This pathway transfers H^+ within a H-bonded network consisting of water molecules and highly conserved Lys, Thr, and Tyr residues [36–42]. In contrast to the D-channel, the K-channel does not seem to have a continuous chain of water molecules;

(C) An extensive H-bonded network between water molecules and the polar side chains of amino acids (Arg, Asp, Tyr, Glu and Asp) has been identified in the extracellular region of bacteriorhodopsin. This network provides a pathway for the transfer of H^+ from the membrane surface to the buried retinal Schiff base [43–45];

(D) Hydrogenases are enzymes that synthesize or consume H_2 . H_2 is essential to the various forms of life that inhabit anaerobic environments [46]. These enzymes, which seem to have been present in the earliest forms of life on Earth, were until recently thought to be present only in anaerobic bacteria whose energy metabolism is dependent on H_2 . Today, it is known that coding sequences homologous with those hydrogenases are widely present among eukaryotes including the human genome [47]. The production of H_2 in Fe-hydrogenases is by the reaction $2 H^+ + 2 e^- \leftrightarrow H_2$. A putative pathway for H^+ transfer from the enzyme surface to the catalytic center in this enzyme ($\sim 12 \text{ \AA}$) was proposed and is comprised of two Glu residues, one Ser residue, and water [48].

H^+ transfer also occurs in many other proteins whose structures are not known in detail like H-ATPases, or not known at all, like H^+ channels [49].

The common motif that emerges from the above descriptions is that of a chain of water molecules inside a protein cavity lined by polar amino acid residues. Evidently, the polar amino acids create a hydrophilic environment. However, this is not the whole story. Because the water wire coordinates with polar groups, thermal fluctuations in the protein structure modulate H^+ transfer in water wires. In Fig. 2, it is exemplified how fluctuations in the protein structure can assist the transfer of H^+ between adjacent water molecules (see legend). Moreover, it is also possible, or even likely in some cases, that H^+ transfer between adjacent waters is actively mediated by polar residues like COO^- or COH . The properties of these transfers however, have not been measured in well-defined experimental conditions. In spite of the truly wonderful structural work summarized above one should be careful when trying to link functional measurements to structural observations. Crystals are

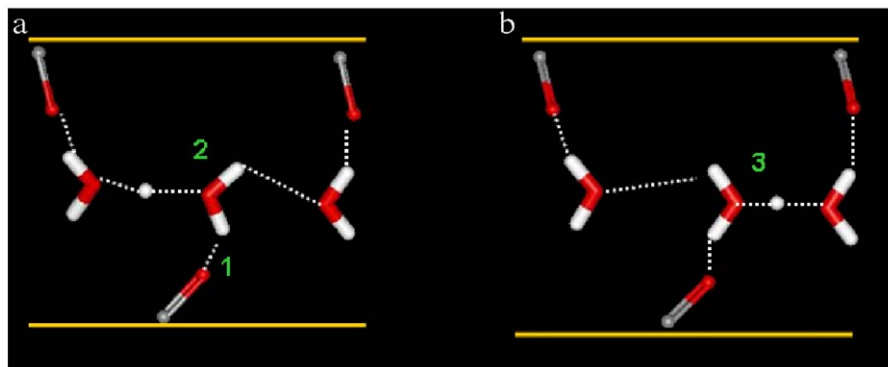


Fig. 2. In this cartoon, inspired by snapshots during MD simulations of H^+ transfer in water wires in gA channels, a simplified example is illustrated in which a H^+ is transferred between water molecules as a consequence of fluctuations in the protein structure. Carbonyls in this figure are shown protruding into the lumen from the protein wall (represented by yellow lines). Waters usually donate H-bonds to carbonyls. One proton is transferred between adjacent waters from the left to the right panel as a consequence of: (a) thermal fluctuations in the protein structure weaken or eliminate temporarily the H-bond between water and carbonyl (1), thus causing the middle water approach the excess proton in the water wire; (b) the middle water would form a new covalent bond with the excess proton (2) releasing one of its protons that will be shared with the water molecule in the right (3). The most direct complex electrostatic interactions between waters, protons, and carbonyls ultimately determine the transfer of H^+ between water molecules in protein cavities. Interestingly, the classical Grotthuss mechanism in which proton transfers are limited by water rotation (see text) could apply to approximately unidimensional water wires, but is it really the rate limiting step?

not grown under the same experimental conditions in which those proteins are fully functional. The characteristics of the most elementary events in proton transfer in water wires in proteins remain poorly or not known.

The properties of water wires in proteins in general like selectivity, rate of H^+ transfer, saturation, and blockade have not been previously addressed. These questions are essential to the understanding of how water wires work considering that (i) water wires select H^+ (10^{-7} M) over other far more concentrated ions (10^{-3} – 10^{-2} M), and (ii) the presence of any ion in the middle of the water would block H^+ transfer, and as such the function of bioenergetic proteins. Moreover, due to the unknown physicochemical properties of hydrated protein cavities, it is quite difficult to ascertain what the rate limiting step of proton translocation inside complex biological structures is.

It has been difficult to address the nature of proton transfer in complex proteins. There are at least two serious conceptual difficulties. First, it is difficult and sometimes impossible to decide whether H^+ are being transferred in one direction or OH^- in the opposite direction (see below). Only in a few cases like in gramicidin channels (see below) is the answer to this question straightforward. Second, experimental measurements of H^+ transfer in biological systems are usually correlated or reasoned in analogy with similar measurements in bulk water which is still the best studied system. This strategy may be problematic because of the specific and unknown physicochemical nature of the H^+ (or OH^-) pathways in a protein cavity which is obviously distinct from bulk water. In this context, it is difficult to define what proton transfer really means. Are protons transferred in a sequence of hopping steps between waters and (de)protonatable polar residues, or is it also possible that a protonated water cluster diffuses in a protein cavity for a relatively short distance? What and where is the rate limiting step for H^+ transfer? Are protons just transferred between water molecules in the water wire? Can polar residues shuttle protons between water molecules? These questions are essential.

Nevertheless the basic experimental parameters (rate of H^+ transfer, kinetic isotope effects, etc.) that would allow an initial response to these questions are not known. The questions discussed above were indeed the major motivation for a methodical study of proton transfer in gramicidin and modified gramicidin channels. As discussed below, these systems were chosen for their relative simplicity.

4. Experimental models of water wires in semi-synthetic gramicidin ion channels

Our goal has been to address the questions raised in the previous section using an initial and simple model based on gramicidin A channels. Native gramicidin A (gA) is a highly hydrophobic pentadecapeptide secreted by *Bacillus brevis*. Its primary structure is an alternating sequence of D- and L- amino acids that in lipid bilayers determines a right-handed $\beta^{6,3}$ helix in which the side chain residues are in contact with the lipid environment, and the carbonyl and amide groups line the pore of the protein [50–52]. The association via six intermolecular H-bonds between the amino termini of two gA peptides, each located in a distinct monolayer, results in the formation of an ion channel [53]. Disruption of these intermolecular H-bonds results in the dissociation of gA monomers and loss of channel function. Of special significance for H^+ transfer is the fact that about 8–10 water molecules [54–56] occupy the entire length (~ 25 Å) of the channel. The channel width (~ 4 Å) does not allow more than one water molecule per cross section and an approximate unidimensional water wire is defined.

Two gA peptides were covalently linked to various molecules [26,57–63]. As expected, the resulting peptide form ion channels in lipid membranes with an average open time that far exceeds those in native gA channels. Our experimental work on H^+ transfer in gA channels has been done with covalently linked gA channels. This strategy was followed because of our interest on the effects of a specific atom or groups of atoms on H^+ transfer in a ‘simple’ water wire as in

various gA channels. These groups can be added to the linker that connects the two gA peptides (see for example [57]) without serious distortions of the secondary structure of the protein that would probably have happened upon changing amino acids. Another advantage is that ionic channels are incorporated into planar lipid membranes and H^+ currents can be easily measured in a single molecule.

The starting point for these studies was the linking of two gA peptides via a dioxolane group [59,60]. Because of the presence of two chiral carbons in this linker two distinct stereoisomers can be synthesized [26,60]. The major distinction between these two dioxolane-linked gA peptides is a localized distortion of the secondary structure in the middle of the channel. This opened the possibility of determining the functional consequences of this distortion on H^+ transfer in the water wires inside the channels.

4.1. Proton transfer in distinct stereoisomers of dioxolane-linked gA channels

Fig. 3 shows energy minimized structures of the SS- (left) and RR-dioxolane linked gramicidin channels. The views in this figure are from inside at the middle of the channel where both gA monomers join the dioxolane. The SS-dioxolane causes a continuous and constrained transition between the two gA peptides. The H-bond network between the amides and

carbonyls lining the pore of the channel is similar to the one in the native gA channel [26,60,62,64]. By contrast, in the RR-dioxolane gA dimer, there is a significant tilt of the linker causing intra- and inter-molecular H-bonds between amides and carbonyls in the middle of the channel to be markedly different from native and SS-dioxolane linked gA channels [64]. Because the channel water wire is H-bonded to channel carbonyls (Figs. 2 and 3) and the patterns of H-bond network are not the same in the dioxolane-linked and native gA channels, it was reasoned that the rate of H^+ transfer would be different in these channels. Fig. 4 confirmed that qualitative prediction and shows the quantitative differences between single channel conductances to H^+ (g_H) in the various gA channels. The SS channel has a linear relationship in the $\log(g_H)$ - $\log[H^+]$ plots in the $[H^+]$ range of 10 mM–2000 M. The slope of that line is ~ 0.75 indicating that H^+ transfer in this channel is *not* limited by bulk diffusion but by the channel itself. The major difference between the SS and gA channels is that in the $[H^+]$ range of 10–1000 mM g_H values in the former are considerably larger than in the latter. On the other hand, the RR-dioxolane linked gA channel has the smallest g_H values among the various gA channels. The shape of its \log - \log plot is distinct from other gA channels as shown in Fig. 4.

At $[H^+] \geq 2000$ mM saturation and attenuation of g_H values occur. The fact that saturation of proton conductivity in bulk water (λ_H) also occurs at high HCl concentrations [25] suggests

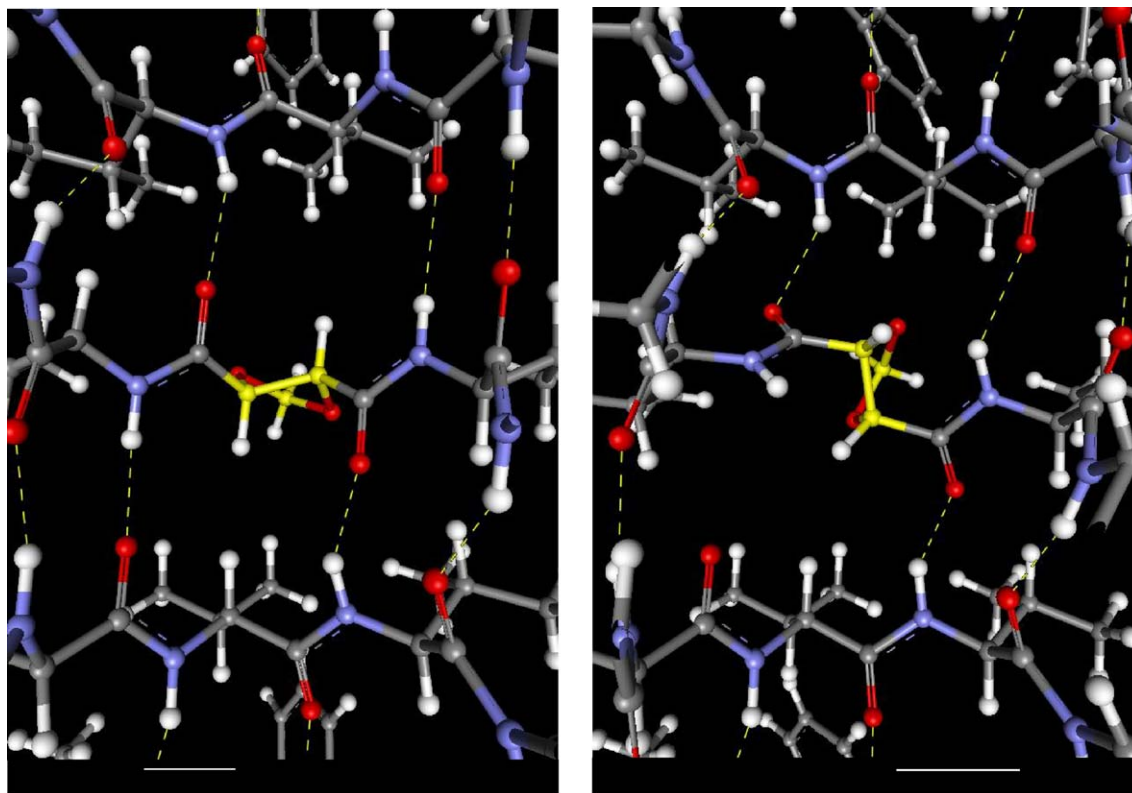


Fig. 3. An inside the channel view at the linkage of two gA peptides via an SS (left) or RR (right) dioxolane linker (from energy minimized structures [26]). C, O, N, H are colored in grey, red, blue, and white, respectively. Yellow carbons are from the dioxolane linkers. Intra and intermolecular H-bonds between CO and NH groups are indicated by yellow lines. While the structural features of those H-bonds in the SS-dioxolane gA channel are similar to the native gA channel, this figure shows that in the RR-dioxolane channel there are pronounced differences in the intra- and inter-molecular pattern of H-bonding [64]. Computational studies indicate that this distortion in the secondary structure is localized in the middle of the channel.

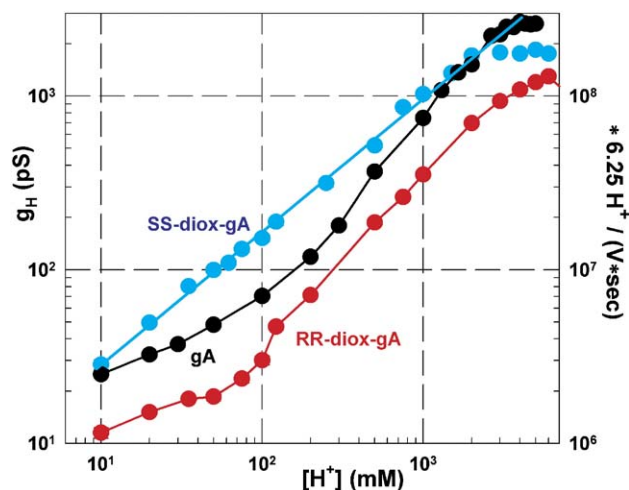


Fig. 4. Log–log plots of single channel conductances to H^+ (g_H in pS) or translocation rates of $H^+/(V \times s)$ across the channel versus $[H^+]$ in mM. Red, black, and blue symbols and lines correspond to RR-dioxolane, native gA, and SS-dioxolane linked gA channels, respectively. Means \pm S.E.M. were plotted in this graph (S.E.M. bars are smaller than the size of the symbols). The straight line fitting the blue circles was obtained from linear regression analysis in the range of $[H^+]$ 10–2000 mM (slope of blue line=0.75). Redrawn from [25].

that this may be part of the explanation for the saturation phenomena in the various gA channels. One possibility is that as $[HCl]$ increases so does the probability of finding a Cl^- inside a water wire with the consequence of blocking proton transfer. As such, the fast transfer of H^+ in solution and *next to the channel openings* is compromised resulting in attenuation of g_H [25]. The number of H^+ inside gA channels is limited and does not change above an unknown maximum as a function of $[H^+]$ in solution. Thus, it is still possible that gA channels may be transferring protons at those high $[HCl]$ (see below) but they could be rate limited by the mobility of H^+ in solution and at the membrane–channel/solution interface. Another interesting consideration is that a H^+ inside the channel may experience long-range electrostatic interactions with the concentrated ions in solution. This would saturate or even promote a decline in g_H – $[H^+]$ relationships at large ionic concentrations [65]. It is not possible to discriminate between this possibility and the ionic cluttering of the solution that makes H^+ transfer unlikely to occur.

It should be noticed that diffusion limitation for H^+ transfer in gA channels (unitary slope in log–log plots of g_H – $[H^+]$ relationships) occur at bulk $[HCl]$ concentrations much lower than 10 mM (Chernyshev and Cukierman, manuscript in preparation).

What are the evidences for H^+ transfer in gA channels? First, gA channels are selectively permeable to monovalent cations only. Fig. 4 shows that g_H increases with $[H^+]$. This is an essential point. In general and in virtually any system, it is not obvious whether protons are being transferred in one direction or OH^- in the opposite direction [71]. Second, g_H is 10^2 – 10^3 -fold larger than for other monovalent cations. In particular, g_H is much larger than g_{Na} which has been assumed to be rate limited by the diffusion of the single file of waters in the channel [66]. Third, kinetic isotope effects ($KIE=(g_H/g_D)$) in various gA

channels at various $[HCl]$ are in the range of 1.31–1.37 [31,67]. In contrast, single channel conductances to various alkalines in native gA channels in H_2O are approximately 10% larger than in D_2O (Cukierman, unpublished observations; [68]). Alkaline permeation in gA channels is accompanied by water diffusion and this must be reflected in the relative small KIE for alkalines. However, interpretation of KIE is not simple [22,23]. Fourth, no electroosmotic potentials develop when H^+ permeates gA channels [55]. Consequently, the permeation of H^+ in gA channels is in significant quantitative and qualitative disagreement with permeation of other monovalent cations. This is taken as evidence for H^+ transfer in gA channels. Taken into consideration the discussion in the previous paragraph, it is not likely that proton transfer in gA channels reflects the hydrodynamic mobility of H_3O^+ inside the pore. This is probably the more general definition for H^+ transfer in gA channels at this time. As to the essential nature of this transfer, much remains to be done both experimentally and theoretically.

In trying to understand the differences in g_H values among various gA channels in the concentration range of 10–2000 mM (Fig. 4), their temperature dependent effects were measured [69]. Table 1 shows the distinct activation energies calculated for H^+ transfers. As anticipated, the Gibbs free energies of activation are not very different among the various gA channels. However, there are significant and major differences between the activation entropies. For the native gA channel, the activation entropy is approximately 25% larger than for the SS channel, and the largest activation entropy occurs for the RR channel. Even though it is not the sole factor, entropic factors could well be responsible for the major differences between g_H in various gA channels.

While the precise molecular origins for differences in proton transfer between the various gA channels are not known, it is possible to explain these differences as a consequence of distinct activation entropies. As mentioned earlier, the transfer of protons is favored by a suitable geometric relationship between H^+ , waters and carbonyls lining the channel wall (see Fig. 2). Thus, the larger the number of possible configurations of these elements the smaller should g_H be. In particular:

(i) in native gA channels, there must be a considerable number of relative distinct configurations between the two gA monomers any of which can provide effective ion channel formation. This could have implications for the structure of the water wire in the channel pore. In some of these water wire configurations, H^+ transfer could be attenuated or blocked in relation to other configurations. Thus, the average rate of H^+ translocation in the channel that is actually measured (g_H) is decreased in relation to the SS-dioxolane linked gA channel.

Table 1
Gibbs free energies, activation enthalpies and entropies for H^+ transfer in various gA channels [69]

Channel	ΔG_o (kJ/mol)	ΔH_o (kJ/mol)	ΔS_o (J/(K \times mol))
gA	26.70	13.11	–45.61
SS	26.59	15.52	–37.15
RR	28.85	12.68	–54.24

(ii) In the SS-dioxolane channel, the two gA peptides are strongly and continuously constrained by the dioxolane. This would have the effect of optimizing the water wire for H⁺ transfer, and as such an enhancement of g_H in relation to native gA channels is measured in a wide range of [HCl] (Fig. 4);

(iii) As for the RR channel, a relatively large number of dynamical conformations of the water wire does not favor H⁺ transfer occurs with the resulting attenuation of g_H compared to the SS-dioxolane linked and native gA channels [69]. A more specific mechanism for this effect has been discussed in MD studies [70]. The electrostatic repulsion between the oxygens in the dioxolane linker and the carbonyls of gA cause a delay in proton transfer in the middle of the channel pore [70]. The more uniform secondary structure along the channel pore that is anticipated for the native gA and the SS channel has the effect of favoring H⁺ transfer in the middle of the channel in relation to the RR channel.

The considerations about the entropic factors above could explain the qualitative differences between rates of proton transfer in the various gA channels. However there is a long way to go in order to have a quantitative understanding of the distinct topologies of g_H -[H⁺] relationships in these channels.

4.2. Some preliminary observations relevant to the above hypothesis. Will Grotthuss history in bulk water repeat in H⁺ transfer in gA channels?

The discussion in the previous section prompted the linking of two gA peptides via a cyclopentane (Narayan, Wyatt, Crumrine, and Cukierman, manuscript in preparation). The major interest on this procedure concerns the replacement of the two oxygens in dioxolane by carbons in cyclopentane. In both SS-cyclopentane and SS-dioxolane, there is a continuous and constrained transition between the linked gA peptides (as shown Fig. 3). Interestingly, the g_H -[H⁺] relationships of these distinct SS channels are basically the same. This supports the view that the basic difference between g_H values of the SS-linked and the other gA channels (see Fig. 4) is a consequence of that continuous and constrained transition that favors the structures of the water wire that are most suitable for proton transfer and/or reduces the probability of finding water wire structures that hamper or delay proton transfer.

By contrast, the replacement of the two oxygens in the RR-dioxolane channel by carbons (RR-cyclopentane linked gA channel) resulted in a significant increase in g_H values. In this case, the g_H -[H⁺] relationship for the RR-cyclopentane linked gA channel became *indistinguishable* from those of native gA channels. These results qualitatively support the idea that the dynamics of the electrostatic repulsion between the oxygens in the RR-dioxolane and carbonyl oxygens may introduce a significant delay in H⁺ transfer in the middle of the RR-dioxolane linked gA channels.

Gramicidin channels have been exerting a special fascination to computational chemists. Because of its small number of atoms the MD treatment of gA channels is not computationally expensive. However, the simplicity of gA channels is often misleading and several facets of gA channels have not been

properly addressed in MD studies. One initial paper on H⁺ transfer in native gA channels suggested (in analogy with the classical Grotthuss mechanism in bulk water, Fig. 1) that the reorientation of water molecules is the rate limiting step for H⁺ [32]. In a posterior study it was demonstrated that the potential of the mean force for water reorientation depends on the water model used in the simulations, and is in semi-quantitative agreement with our experimental measurements [70]. A recent development was the proposal that the electrostatic barrier for H⁺ transfer and *not* the reorientation of water molecules is the significant rate limiting step for the permeation of H⁺ in gramicidin channels [72,73,75]. However, and in view of the significant entropic component of proton transfer in the various gA channels discussed above, it is not clear what the effect of applied energy constraints in MD simulations would have on the free energy of water reorientation.

A distinct problem that has not been addressed in this review is that proton transfer in gA channels is *heavily modulated by specific phospholipid headgroups and acyl chains* ([74]; Chernyshev and Cukierman, manuscript in preparation). The absence of a detailed representation of lipid membranes in MD calculations means that we are far from providing a “realistic” answer to the questions addressed in those studies in spite of claims in this direction.

The simple experimental models that we have been developing face an extraordinary challenge for computational studies. Our experimental models show a difference of 2–4-fold in the rate of proton transfer among distinct covalently linked gA channels (Fig. 4), and a 10-fold difference if the effect of lipid membranes are considered ([74]; Chernyshev and Cukierman, manuscript in preparation). These rate differences are easily measurable, reproducible and their standard deviation are small [25]. However, when those measurements reflect changes in activation energy of a modest 0.7 kcal/mol (or 1.4 kcal/mol in the case of membrane effects) difference between the fastest and slowest proton transfer rates. These values are comparable to the thermal noise itself and unlikely to be unraveled in MD studies unless there are major localized changes in the rate of proton transfer.

The development of scientific hypothesis is a consequence of time and maturation of ideas rather than the desire to provide quick and superficial answers. The rate limiting steps of the high H⁺ mobility in bulk water and in models of water wires in simple gA channels remain a challenge. However, it is easy to ascertain that major progress in the last 10 years has been made: the rediscovery of the proton mobility problem, the new insights to an uncritical century old model that has been extensively patched up as discussed here, and the vigorous experimental and computational reinvestigation of proton transfer in various systems. Eventually, new insights will bloom.

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