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WATER, PROTON TRANSFER, AND HYDROGEN BONDING IN ION CHANNEL GATING

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1. ABSTRACT

Several types of ion channels, the proteins responsible for the transport of ions across cell membranes, are described. Those of most interest are responsible for the functioning of nerve cells, and are voltage gated. Here, we propose a model for voltage gating that depends on proton transport. There are also channels that are proton-gated, of which some are bacterial. For one, a structure is known in the closed state, the KcsA channel (1). The proton gating of this channel suggests a part of the overall gating model we propose. Other bacterial channel structures are also known, but none that are relevant here, at least in one case because it appears to be in the open state. Voltage-gated channels of eukaryotes open in response to the depolarization of the membrane. It appears that there is some analogy in the structure of the voltage-gated channels to the structure of the smaller bacterial channels, including the one that is proton-gated. There is also significant experimental work in the literature on the nature of the gating current, a capacitative current that precedes the opening of the channel. The model we provide is based on the known properties of channels; in this model, voltage gating consists of three stages: first, the tunneling of a proton as depolarization begins, to initiate the sequence; second, proton transport along a sequence of (mostly) arginines, which is postulated to bring a proton to a critical gating region, where, third, a strong, short, hydrogen bond is weakened by the added proton, allowing the four domains to separate. The separation of the domains allows ions to pass through, and thus constitutes the opening of the channel. An analogy to the behavior of ferroelectrics is also described.

2. INTRODUCTION: ION CHANNELS

2.1. What are ion channels, and why do we care?

Ion channels are proteins found in plasma membranes of cells, as well as organelles; channels are responsible for the transport of ions across the membranes. They are ubiquitous, but some, those found in "excitable tissues", which include neurons, are of particular interest. These include, for example, the sodium and potassium channels that are responsible for the transmission of the

nerve impulse. Every living cell, however, must have channels in order to control the traffic of ions through the cell membrane, and thus to control the composition of the cell.

The structure of these channels is not known, but the structure of some of their analogues in bacteria have been worked out. Generally the bacterial analogues contain only the central part of the relevant channel structure, which makes them easier to crystallize, and thus makes it possible to have their X-ray structure determined.

The channels must exist in a closed form, in which ions are not transported, and an open form, in which they are. While the channel is undergoing transitions leading to opening, it passes through a number of closed states that are not the same as the original closed state, but are stages on the way to opening. Some channels may have more than one open state as well, the states distinguishable by their ionic conductance. However, most of the channels with which we shall be concerned have only one conducting state. In recent years, a great deal of progress has been made in answering two of the main questions concerning channels: how do they conduct, and how do they select one ion over another? The structures of the bacterial channels have made it possible to solve these problems reasonably well, as the structures include the critical sections that govern these aspects of channel function. The third question, the nature of gating, the process by which channels open, remains unsolved. We will present a model for this process here.

2.2. A possible model for gating

There is a fair amount of evidence that protons may be involved in gating. This includes the gating of one of the bacterial channels, KcsA, in which a drop in pH opens the channel. It also includes calculations on voltage gated channels, or at least models of them, and qualitative comparison with a number of experimental results. This review will be largely concerned with this set of questions. In order to deal with them, we will consider the tunneling of protons in a model system, the transport of protons in the appropriate model, and the use of the KcsA channel, as a possible model for the use of protons in opening a channel by weakening hydrogen bonds; the hydrogen bonds, in this model, are responsible for keeping the channel closed. Weakening these bonds allows the channel to open. In order to consider the facets of the model, we will have to take a tour through what is known about ion channels, as well as some of what is known about proton transport, and the evidence that may tie channel gating to proton transport.

2.3. The basic structure of some ion channels

Because the channels with which we are particularly concerned, the sodium and potassium channels of nerve and other excitable tissue, have analogues in many other tissues, ranging back to bacteria, we can look at the structures of these bacterial channels first; while it is not always true that the function of a protein can be deduced from the structure, here the structure is extremely informative in understanding how these channels function. Potassium channels in particular have been recently reviewed by Yellen (2). Fundamentally, there are a set of transmembrane (TM) segments that are arranged in four domains. For potassium channels, these are tetrameric, while for sodium channels they are not exactly tetrameric, but the four domains are very similar. Variations on the architecture are sometimes found, but a central pore formed by four domains appears to be a consistent theme. The bacterial channels have only two TM segments per domain, for a total of eight around the pore, and in fact only four segments actually create the pore. The first structure of such a channel, the KcsA channel of S. Lividans, was published in 1998 (3); since then, a revised version has been published (1,4). This appears to show, at high resolution, the structure of the closed state of a K^+ channel with two TM segments per domain, plus a pore lining segment that forms a linker between the two transmembrane segments. Another channel, MthK, with 2 TM segments per domain, has been determined (5). This structure appears to be open; the domains show a wide separation at their intracellular ends, instead of a tight configuration as in the KcsA case.

Eukaryotic channels generally have 6 TM segments per domain (although many varieties exist). The channels with which we shall be primarily concerned are of the 6 TM variety. However, there are some channels with double pores, and 2 x 2 TM segments per domain, some with unusual properties that have 6 + 2 segments, among other related varieties. However, all potassium channels have the same conserved structure in the selectivity filter (see Figure 1). There are highly conserved G-Y-G residues in the linkage section between the two TM segments of bacterial channels, or in the corresponding sections of eukaryotic K^+ channels. The G-Y-G sequences of the four domains appear to replace the water of hydration of the potassium ion with their carbonyl groups, making an almost exact fit, energetically, while failing to do so for sodium, for which the fit is poor. Hence these channels have a high selectivity, often around 10^3:1, for potassium over sodium. In this way, the structure of the channel shows how one of the major problems in understanding channel function is solved.

Another major problem is the conductivity. Here, there have been a number of molecular dynamics (MD) studies of the KcsA and MthK channels. As the pore is similar to that of the eukaryotic channels, it is likely that the same considerations apply to those. Here, the question is how the ion is dehydrated, then rehydrated, and moved through the channel with about the same mobility as it has in aqueous solution. It turns out that the channels have, especially in the open state, a significant amount of water in the pore. The water may be largely oriented, especially while the ion moves through. Three ions appear to be in the channel at a time, and ions moving through displace those ahead of them. The MD simulations show adequate conductivity in the channel to produce the observed currents.

There is a third main problem: gating, the opening and closing of the channel. This problem continues

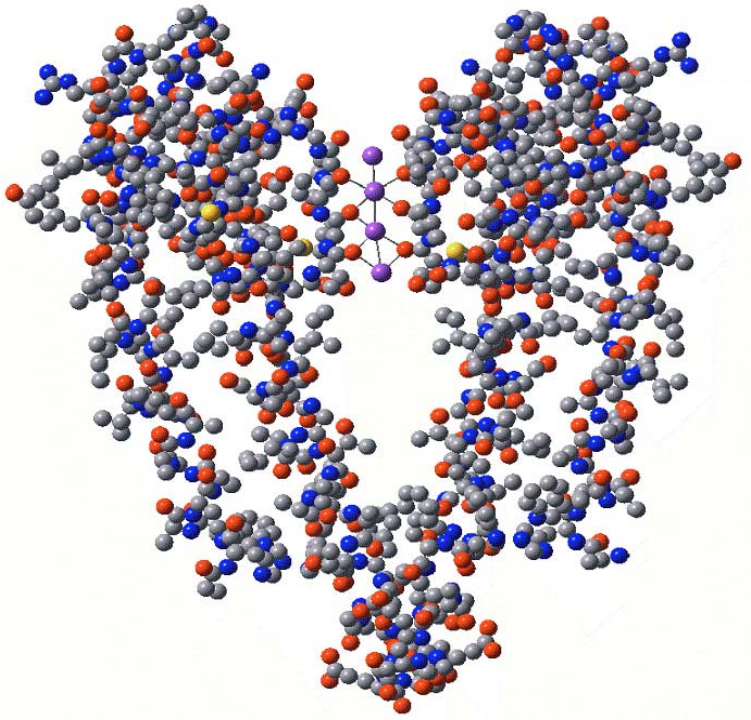

Figure 1. The KcsA channel, using the 1j95 coordinates (1). The figures are created using Gaussview®. Only C (gray), O (red), and N (blue) atoms, as well as a few S (yellow) atoms are present, plus 4 potassium atoms (magenta); hydrogen atoms, and all water molecules, are missing. Two domains of the entire structure are shown, with two omitted for clarity: the two omitted domains would be in front of and behind the two shown. Observe the selectivity filter in the upper (extracellular) section, in which four potassium atoms are shown complexed by oxygen atoms. In the real channel almost certainly only two of these positions would be occupied, and there could be an additional K^+ in the "pool" defined by the bulge below the selectivity filter. This space is about the size of a hydrated potassium ion, and can contain water, which would almost certainly be oriented by the walls in the absence of potassium.

to pose the most interesting questions; it is also the key to understanding the control of the channel, thus of the determination of the timing, for example, of the nerve impulse.

2.4. Gating of voltage gated potassium channels, the corresponding voltage gated sodium channels and related bacterial channels

Here we consider gating by voltage in eukaryotic channels, and proton gating in the related KcsA bacterial channel. There are two aspects to the question of gating. One is the matter of the actual conformations of the protein in the open and closed states. The other is the means by which the channel gets from one to the other. The structures give a strong sense of what the open and closed states look like, with the KcsA channel apparently closed, the MthK channel apparently open. In the closed state the TM segments of the four domains come close near the intracellular end, forming a "bundle crossing" that is usually regarded as the place where the ions are blocked, closing the channel. The bundle crossing leaves an opening that is quite small. However, there is no direct evidence that shows that this actually is the location at which passage of the K^+ ion is blocked; the gate and the bundle crossing may be in different locations. In the KcsA channel, the bundle crossing appears to be around residue 108, while critical gating mutations seem to occur not only there, but especially in the 117 to 121 region, about 10-15A more to the intracellular side. It is also possible that the mechanism through which the channel is unlocked may not be in the same location as the block, so the block may be in the bundle crossing and the locking mechanism elsewhere, making a two part gate.

It has been proposed that, given the conservation of structure of potassium channels, the gating mechanism, and general structure of potassium channels, is similar in the bacterial and in eukaryotic channels (2,5). There appears to be a hinge in the protein that allows the closed section to swing out, producing the large diameter pore. The hinge is not always in exactly the same location, although in the eukaryotic channels (but not the bacterial channels) it generally seems to involve at least one proline, or, more often, glycine. The swing in the KcsA channel, based on ESR evidence, seems smaller (6,7).

What is not known is the means by which the gating stimulus is coupled to the channel opening mechanism. The KcsA channel gates upon lowering pH, thus presumably with the addition of a proton. The channels that have 6 TM segments instead of 2 gate with voltage, or else with a ligand that may have a binding site

in the C-terminus of the channel. The TM segments are labeled S1 to S6, with S5, the pore linker, and S6 constituting the analogues of the two TM segments of the KcsA channel. S6 is the main pore lining TM segment. S1 to S4 are present in the eukaryotic voltage gated channels, but not the bacterial channels. While we will not discuss ligand gated channels nearly as much as the voltage gated channels, they may also have a similar mechanism for opening.

The question then is, how does the proton open the KcsA channel? Is the voltage gating mechanism, which apparently requires the four additional TM segments, a form of voltage to proton current transducer, in which the four outer segments respond to the drop in membrane polarization voltage by starting a proton cascade, bringing the protons to a location at which they weaken one or more hydrogen bonds? If so, the final step in the gating mechanism of the voltage gated channels could be similar to that of the KcsA channel. We will argue that this is the case.

KcsA opens by rotating its pore lining segments outward. Gating of KcsA by a proton suggests the question of how a proton forces the channel to rotate. However, there is an alternate possibility to forcing the channel open: the channel is held tightly in place in the closed state by hydrogen bonding, with the added proton(s) releasing, not forcing, the bonding. The channel then relaxes to the open state. New hydrogen bonds are needed to form the closed state again. When the internal pH is lowered, the KcsA channel presumably receives one or more protons, titrating, in all likelihood, a set of glutamate residues (numbers 118 and 120 in the Zhou, et al structure (1)). We propose that the voltage gated channels drive a proton gating current within one of the additional TM segments, S4 (see below), to provide the proton(s) needed to allow the same effect to open the channel. It is known that S4 can, under some conditions, conduct protons (8-10). To understand how this happens, we need to consider what is known about the motion of protons in proteins and in water, and about short, strong hydrogen bonds (SSHB). Before we do this, however, we should consider how S4 has been looked at so far, in relation to gating.

S4 has positive charges every third amino acid, mostly arginines. There is no doubt that S4 is involved in gating, as this has been demonstrated by extensive mutational experiments. There is one type of experiment, performed over the years by a number of groups (for an excellent example involving sodium channels, see work by Horn and coworkers (11)) that has led to the suggestion that the S4 segments must move extensively in gating, possibly dragging some part of the pore along. Upon mutating the arginines to cysteine, and then using methanethiosulfonate (MTS) reagents, which react with the –SH groups of cysteine, it appears that certain locations may be accessible intracellularly when the channel is closed, and extracellularly when it is open. The implication drawn by Yang and Horn, and others, is that the segment moved across the membrane, exposing residues to different sides in the open and closed states. As the reagents are large, there must be some aqueous space around them. However, access restrictions can happen in more than one way, for example, with bound water blocking access from one side or the other differently in the two different states; this could produce the differential access results without any movement of the S4 segment. In an attempt to obtain a better indication of the extent of movement, experiments were done by Bezanilla and coworkers in which fluorescent tags have been placed on the several amino acid positions, and the relative positions determined from fluorescence energy transfer (12). The results rule out large S4 motions, especially perpendicular to the membrane, as would seem to be implied by the accessibility results. These authors have proposed (13) a scheme in which rotation of the S4 segment could preserve the distances, and thus account for the fluorescence energy transfer results. The accessibility data would be preserved by turning the segments to face aqueous crevices that extended from one or the other surface of the membrane. The fluorescence transfer results also suggest a maximum movement of 3A for any residue with respect to S4, but that is for one residue in the S3 – S4 linker; removing this linker still allows the channel to gate. The other residues move at most about 1 A. The relative movements are small, and the absolute movements could be also if water blocked the aqueous crevices differently in the open and closed states (there must be some access for the reagents to react, which they clearly do). Bound water blocking access seems to us the simplest explanation.

There are salt bridges between S4 and residues in the S2 and S3 TM segments (14). The energy associated with the salt bridges must be compensated in some manner, either by forming new bridges as the old are broken, or by motions in which the bridges are preserved. The Bezanilla model is probably the best of the models that propose a relatively large scale motion to account for the coupling of voltage to gating. It would mean that unlike the selectivity, there would be a completely different mechanism for the gating of the bacterial and eukaryotic channels. As with all other models of gating, calculations of the energetics will have to await the determination of a structure. However, it looks as though there would be a great deal of difficulty with any model that requires large scale motion of the S4 segment, for energetic reasons among others. Since there presumably is appreciable motion of the pore-lining parts of the channel, these motions must couple to S4 by some other means, possibly electrostatic, or possibly by way of hydrogen bonding, as we will suggest.

Finally, we note that very recently a new gating model has been put forward by MacKinnon and coworkers, based in part on an X-ray structure that includes a voltage-gated channel from an archean, which nevertheless was shown to be very similar to eukaryotic voltage gated K^+ channels. (15,16). However, the crystal structure appears to be determined more by the attached antibody than by the channel, and some experiments appear to be seriously incompatible with the gating current model, so we will not refer to this model(15,16) further.

2.5. Other channels, and how protons influence their gating

2.5.1. Some special channels

There are other, specialized, channels, that may gate, for example, upon stretching. In addition to the major gating stimulus, gating may be modified by ions present in

the medium, and in particular by protons. Certain eukaryotic channels gate in response to protons directly (17). These are present in the nervous system of higher animals in nociceptive neurons, for example. In addition to these, and the KcsA channel, protons are known to influence the gating of voltage gated ion channels in the central nervous system (18), as well as of ligand gated ion channels (19).

For most channels, there is one, or at most a few, conductance levels, and these are fixed by the structure of the channels. The total charge that crosses the membrane is determined generally not only by the conductance, but by the channel opening frequency and time. The control of this time therefore controls the functioning of, for example, nerve cells. It also must be tightly regulated to insure that the concentration of ions in the cells is within physiologically acceptable limits.

There are additional families of channel-like entities that function as antibiotics or are produced by bacteria for other reasons, and they may not have either structure or function comparable to those channels that concern us here. They do have transport properties that may be similar however, and they may transport protons. The most studied, gramicidin, is discussed in this issue by three authors (Cukierman, Schumaker, and Pomes and Yu).

In addition to the TM segments, there is a significant fraction of the protein in potassium and sodium channels that extends beyond the membrane, in the N and the C terminal regions. The role of these sections of the channels is less well defined, especially from the point of view of gating, although very important from the point of view of inactivation. Inactivation is a process that closes the cell to ions without restoring the original closed configuration from which the channel opens. Returning to the fully closed state is apparently relatively slow, so inactivation allows the current to be cut off in time. In voltage gated channels, inactivation is accomplished by two mechanisms, one of which is fast. That one depends on a positively charged structure near the N-terminus of the protein. If this structure is thought of as a ball, it fits into a socket in the channel entrance and blocks the channel. (The geometry is not all that spherical, but metaphorically, the ball and socket is a good description.) There are four such structures in the K^+ channel, and one suffices to block (inactivate) the channel. The other mechanism may involve the selectivity filter, and appears to be the same as "C-type" inactivation, which is slower. We will not discuss this here.

2.5.2. Ligand gated channels

The structure of some ligand gated channels is quite different from that of voltage gated channels. There are ligand-gated potassium channels that maintain a similar overall structure to that of the voltage gated channels discussed above, such as the CNG channels (cyclic nucleotide gated, gating in response to binding a cyclic nucleotide in the C-terminal region of the channel), but there are other entire families of channels. For example, instead of a tetramer of 6 TM segment domains, there are five segments in many of these channels, such as the AChR receptor, which includes a sodium channel. Each segment has 4 TM α-helices We cannot give a comprehensive discussion of all ligand gated structures, but it is worth considering whether these may be functionally related to the rather different structures of the voltage gated channels we are primarily concerned with.

The AChR receptor structure is known to about 4 A resolution at present based on electron crystallographic methods (20). What is interesting is that the structure still has some major characteristics of the voltage gated channels, and even of the KcsA channel: that is, there is a constriction at each end of the channel, with a wider section between. Binding of two acetylcholine ligands in the AChR, and binding of other ligands in other channels, leads to a distant conformational change (allosteric change) in the remainder of the channel leading to channel opening. The nature of this change has yet to be defined for any channel. The channel has five subunits, with two segments to a subunit. There is a gate for the resting state, possibly near the intracellular end between segments M1 and M2 of the α subunit (of the five major subunits of the channel, generally two are of α type, and these are two segments of this subunit); the gate in the desensitized state is further up, (21), or possibly further up in the resting state as well (20). The lumen has a pore with a wide section, somewhat reminiscent of the pore in the voltage gated channels. The structure of this channel is better known than that of other ligand gated channels, and it is interesting to note the apparent general functional similarity to the voltage gated channels. For this channel, there is a bell-shaped dependence of gating on pH (22-24), but the pH dependence of gating has been studied less thoroughly than it has been in some other ligand gated channels (19).

2.5.3. Gating with signals other than voltage or ligands

Other channels have other gating mechanisms, or at least respond to stimuli other than voltage, or ligands, and pH is not their primary signal for gating, although it may affect gating. These include TREK channels, and the MscL channel of certain bacteria, which gate in response to stretch. TREK channels also are affected by pH (25,26). They have a slightly different structure, with 4 TM segments and two pores. McsL channels are homopentamers with two TM segments per domain, for a total of 10 TM segments. The closed (27) and open (7) conformations have been worked out, and show a huge opening, with the passage increasing in diameter from approximately 3.5 A to over 30 A when it gates.

This list only gives two examples, and is obviously not exhaustive.

2.5.4. Two classes of pH dependent channels

There are two other large and important classes of channels that have their gating significantly affected by pH. These include the K_{ir} (potassium inward rectifier) channels that are important, for example, for potassium secretion and NaCl readsorption in the kidney. These channels gate in response to a combination of extracellular K^+ and intracellular H^+, which apparently interact allosterically (28,29). In addition certain Ca^{2+} channels have gating that responds to pH.

All these classes have some pH response in their gating, or else, as in the case of the voltage gated K^+ and Na^+ channels, they have properties that respond to H^+ ions. However, the responses are not always mediated by the same mechanism. Neither is the response to protons necessarily the primary gating mechanism for all types of channels, but there are certain possible similarities. We will consider primarily the mechanism we are proposing for the gating of the K_v family of voltage gated potassium channels, and the related Na channels. The fact that other channels show pH effects must be noted; it is also possible that these effects have some relation to those found for the channels of primary interest, but we cannot know this until a structure is found.

2.5.5. Acid Sensitive Ion Channels, and their relatives

Finally, there is an entire class of proton-gated channels, primarily for Na^+, but not so ion selective, which are found in mammalian species, the worm C. Elegans, and various other species (30-32). These are the ASIC (Acid Sensitive Ion Channels), and belong to the same group as ENaC/DEG channels (these seem to be largely the same type of channels, albeit with different names in different species). We do not know much about the structure of these, so we cannot discuss them at length. However, they are clearly physiologically important, and the fact that they are proton-gated suggests that they will be of considerable interest for us.

3. WATER, HYDROGEN BONDS, AND PROTONS, IN ION CHANNELS

3.1. Water

Water in an ion channel is somewhat different from water in bulk. As a consequence, some of the characteristics of water in channels are not well represented by the standard point change models that are used in molecular dynamics (MD) simulations. It has been shown in numerous simulations, both MD (33,34) and Monte Carlo (MC) (35,36) that water in a small pore will line up parallel to the walls of the pore. Other workers have found similar effects (e.g.,(37)). Furthermore, a high electric field also helps to align water. These are the principal reasons water in the central cavity of the channels is unlike bulk water. The extent of the effect is dependent on the channel walls and the electric field present. A detailed understanding is difficult to achieve without a detailed structure. There is evidence supporting the existence of a high electric field; this includes experimental work by Pascual and Karlin on the AChR channel (38), who showed a field at least of the order of 10^8 V m^{-1} could be inferred from the rates of reaction of methanethiosulfonate (MTS) reagents that were attached to cysteines in the channel (the technique of mutating various residues to cysteine and then reacting with MTS reagents to determine accessibility was developed by Karlin and Akabas. (39)). The Pascual and Karlin result was obtained by comparing the rates of reaction of positively and negatively charged residues with MTS reagents in the channel. . (The recent finding of a high field in a section of the S4 segment of Shaker (40) is not relevant, as we are concerned here with the pore.) Certainly high fields are plausible, based on calculations for a pore (35), and for the photosynthetic reaction center (41,42). Furthermore, in one case, it has been shown that changing the charge on the channel (in this case, a wide channel from a bacterium, capable of transmitting polymers, unlike any of the selective gated channels discussed above) does alter the conductivity of the channel. This has been ascribed, quite reasonably, to the binding of water to the channel wall (43), thus constricting the available space for the conductivity of the polymer which was used as the conductivity probe. Even though this was in a channel of one of the larger types, the properties show that water does behave in a way that is different from bulk. A smaller channel would be expected to show an even stronger effect, albeit not so easily measured. An estimate of the contraction of the effective channel diameter, based on the radii of the polymers in solution, suggests that there is about one layer of water molecules adsorbed on the wall of the channel sufficiently strongly to obstruct the passage of the polymer.

While ordinary MD or MC simulations suffice to demonstrate the alignment of water in a pore, more complex phenomena are encountered in a channel, where the wall structure is important. This is particularly true of hydrogen bonding. Hydrogen bonds come in a variety of lengths and strengths, something that cannot be reproduced by point charge models, so that quantum mechanical treatment is required.

3.2. Hydrogen bonds

Hydrogen bonds can be approximately categorized as normal or "strong, short" (SSHB). There are gradations within the categories, and the term "low barrier hydrogen bond" (LBHB) is also used. There are actually differences in the definitions of SSHB and LBHB, but we will consider them together as a single category of bonds that are stronger than normal, and shorter, regardless of whether there exists a barrier to the transfer of the proton between the two heavy atoms (which may be oxygen or nitrogen, for the cases in which we are interested). For the moment, SSHB will be understood to include LBHB. There are also intermediate strength and length bonds, but normal and SSHB types are adequate for the present discussion. We will see below that the bonds are capable of switching between SSHB and normal as a parameter of the system is changed, and we wish to suggest that this is relevant in ion channel gating.

The difference of the bond length generally is a bit more than 0.2 A. SSHB are generally less than 2.5 A in O – O distance, for example, while normal bonds are at least 2.7 A. The difference between bond strengths can be of the order of more than 10 kT, sometimes 20 kT, where kT is thermal energy, so that they have thermodynamic significance. A variety of SSHB have been proposed to account for the effectiveness of certain enzymes, and catalytic effects up to 10^5 have been attributed to these bonds. (44-46).

There is as yet no direct experimental evidence for the existence of any sort of hydrogen bond in channels, including SSHB or LBHB. It has been proposed, on the

basis of a quantum mechanical ab initio (actually, density functional) calculation that a SSHB may be responsible for holding together the closed state of the KcsA channel (47). The key formation is an $H_5O_2^{\delta+}$ group that has a partial charge, shared with surrounding groups; this is the reason for the δ+ charge, as opposed to a simple +. The group differs from the Zundel ion, $H_5O_2^+$, which is a group in itself, although existing in bulk water (48,49). The group will be discussed in more detail below. However, confirmation awaits further investigation. Other theoretical work on biological implications of SSHB is becoming extensive, although concerned with enzyme reactions rather than channels. Schiott *et al* concluded that all such systems possess similar electronic structures, with polar covalent bonds between the hydrogen atom and the two neighboring heteroatoms (50). Northrop has shown how a proton in a low barrier hydrogen bond, shuttling between oxygens of aspartate carboxyl groups, holds the carboxyls coplanar. The proton shuttles by tunneling, and is not transferred to substrate (51). There are many cases of proton transfer, some of which are directly relevant to enzyme catalysis.

3.3. Proton transport and tunneling in gating ion channels

3.3.1. Proton transport

Proton transfer in ion channels (other than gramicidin — gramicidin is discussed elsewhere in this issue (Cukierman, Schumaker, and Pomes and Yu), as are certain channels specialized for the purpose of transporting protons (see, in this issue, Morgan and DeCoursey)) has not been experimentally demonstrated in the normal functioning of channels of the types we are interested in, in which there is a central pore. However, the S4 TM segment, which has been shown to be central to voltage sensing, transports H^+ in a mutant of the much-studied Shaker potassium channel. The nature of the proton current is particularly instructive, as this segment is known to be central to gating not only in that channel, but all other similar voltage-gated channels. When the last two (most extracellular) arginines in S4 are mutated to histidines, the S4 segment conducts protons, while mutations of other residues do not produce this result (8-10). We will argue that gating current in these channels is in fact a proton current, and this experiment provides evidence that such currents can exist. Theoretical work on proton transport generally is discussed elsewhere in this issue by Voth.

There is evidence for analogous currents in other systems, including certain enzymes. Bacteriorhodopsin, also discussed elsewhere in this issue (Luecke), has a somewhat similar current, except that there the arginine residues maintain their charge. Murata and coworkers have suggested, based on ab initio calculations on a section of bacteriorhodopsin (BR), that a water molecule is required for stability in the formation of the key protonated Schiff base (52). A similar experimental result was obtained by Maeda, based on FTIR spectroscopy in both BR and bovine rhodopsin (53). Bacteriorhodopsin has also been found to have liquid-crystal like ferroelectric properties (54), an interesting observation from the point of view of the apparent analogies of ion channels and ferroelectrics discussed below.

We have done ab initio calculations on the transfer of a proton between two arginine residues, finding the PES and minimum path for the transfer. A path can generally be found if there are enough water molecules; the molecules rotate as the proton passes, handing it off to the next water as it goes by. Section C1 below describes this work in more detail.

3.3.2. Tunneling

There are also calculations pertaining to enzymatic reactions in which proton transfer, apparently by tunneling, is a rate limiting step, or is otherwise central to the mechanism. Three such cases were studied by Truhlar, Gao, and coworkers (55). These included enolase, in which quantum effects were required to understand the Kinetic Isotope Effect (KIE), and in which there is a tunneling contribution, alcohol dehydrogenase, in which there is also a large tunneling contribution that must be included to understand the KIE, and methylamine dehydrogenase, which has a very large KIE, with significant vibrational free energy and tunneling contributions.

Other cases in which proton transfer involving tunneling has been discussed theoretically include a gas phase formamidine and its monohydrate complex (56), and intramolecular proton transfer of the hydrogen oxalate anion. [57]. A new hybrid approach to including such effects in MD calculations has been proposed by Billeter *et al* [58]. More general brief reviews of tunneling have appeared for aspartic proteases (51), and for tunneling in biological systems (mostly enzymes) generally (59). Each cites numerous experimental references. One expects, in tunneling, to see an extremely rapid transfer, which looks like a small, fast current. A phenomenon that very nearly seems a signature of tunneling appears to be the first step in gating the Shaker channel. Stefani and Bezanilla (60,61) have found that the first step in gating current is a pulse of less than 2 μs (too short to resolve) and about 1% of gating current.

All told, a fair number of cases of proton transfer involving tunneling, some with SSHB in some form, have been studied by ab initio methods. They include a variety of enzyme systems, showing that there is nothing peculiar about mechanisms that include such bonds. So far, there has not been much experimental work on proton transport directly relevant to ion channels, the main exception being the work of Starace and Bezanilla, cited above, showing a proton current through S4. In addition, Stefani and Bezanilla's demonstration of the initial fast current appears to be relevant to proton tunneling, but has not been demonstrated to be such. The putative binding of carboxyl groups of E118 in the KcsA channel (62) is the main theoretical or modeling attempt at using this phenomenon to explain gating. However, based on the newer (1J95 (1)) coordinates, it is Q119 that holds the domains together. In the new structure, the Q119 residues form a ring of N and O atoms within hydrogen bonding distance; see Section 4.1.

The other form of experimental work that could be directly relevant is on deuterium substitution, on which

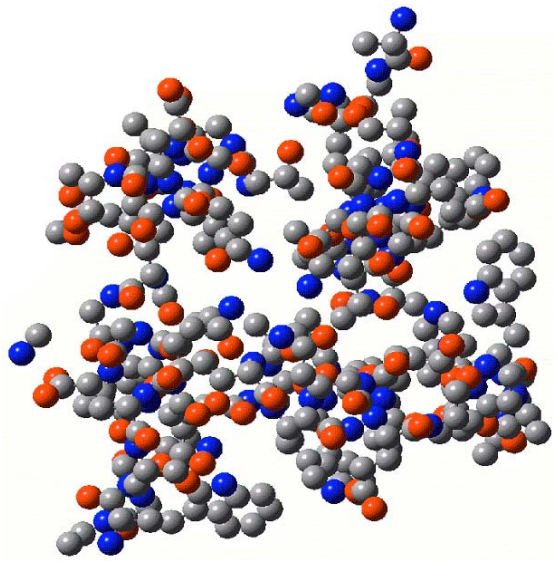

Figure 2. The section near the gate: this is shown from beneath (in the coordinates of Fig. 1), with all four domains represented, and only residues 117, 118, and 119 of each shown. Note the four nitrogen atoms (blue), belonging to Q119, forming a square. This group is tightly hydrogen bonded (see Table I). The amino acid sequence for each domain is REQER, (the last ER are not in the figure) so that each gln (Q) is surrounded by two oppositely charged residues on each side. This appears to be the region that is most crucial for gating, judging from the mutation results of Perozo (private communication). The charged residues presumably could also be titrated by a proton when pH drops. Also, if the ring of nitrogens is crucial, then a mutation of that ring should have drastic effects. One might expect that Q119A (for example—some other mutations of Q119 would be similar), if it expresses at all, would be constitutively open, as it would lack the hydrogen bonds needed to hold it together. In the 1j95 coordinates, the guanidinium groups of the arginines are not given (hence, are absent from the figure), so that they are presumably mobile in the crystal structure; their charges are important, but not their positions.

there have been a number of physiological studies, by Schauf and coworkers (working with Myxicola) and Alicata, *et al* (working with crayfish) (63-67). The gist of the results is that there are two major sections of the gating sequence: the initial gating current is not affected significantly by deuterium substitution, but the final opening step is. The deuterium effect is larger at low temperature than at higher temperature (compare 4^o C to 18^o C). No particular differences were found for the two types of organisms.

4. ION CHANNEL GATING: AN HYPOTHESIS

We have looked at several phenomena so far that relate to proton transfer: SSHB, proton tunneling, and proton transport in a model of the S4 segment. We will see that these are the elements needed to produce a model for ion channel gating. The channels we are particularly concerned with in this model are voltage gated potassium channels, such as Shaker, but the model should apply also to sodium voltage gated channels.

The hypothesis is that gating depends on the arrangement of four domains with titratable residues close enough together that they can be held by SSHB in the closed state. Proton transport is triggered by tunneling of a proton between two amine, or guanidinium, groups. The transport in turn brings a proton to the titratable residues, which are expected to be accessible to water. The water at the gate is for the moment hypothesized to be two waters plus a hydrogen which together form a SSHB; a network of hydrogen bonds including the SSHB is a possible variant of this model. With a new charge added by transport, the SSHB weakens to form a normal hydrogen bond. This cannot hold the domains together, and they fall apart, opening the channel.

4.1. Hydrogen bonds in the gate

This hypothesis implies that the channel is expected to be relaxed in the open, rather than the closed, state; the closed state is held together by tight hydrogen bonds, while the open state remains a lower free energy state until the tight hydrogen bond forms again. The weakening of the SSHB allows the domains to relax to the open state. There is no need for the block of the K^+ ions in the channel to occur at the same place that the hydrogen bonds hold the domains together; the ligation of the domains can occur far from the bundle crossing of the domains, while the block of the K^+ ions may still occur at the bundle crossing. This is possible because the side chain of the group thus ligated may extend from the backbone into the center of the channel. It appears that in the 1J95 KcsA coordinates, the Q119 residues do this, producing a set of hydrogen bonds with their own side chain amide nitrogens and oxygens. (See Figure 2). The nitrogen and oxygen atoms of the amide side chains are spaced to make good hydrogen bonds (albeit not SSHB) around the ring; the distances are given in Table 1. It remains to be seen whether the water in the neighborhood produces additional SSHB; to calculate this with the requisite accuracy requires optimizing a rather large number of atoms. Work along these lines is progressing, but will need much time to complete. Based on these coordinates, it appears that a Q119A mutation (for example) would lead to a constitutively open channel, and the same might hold for a mutation to other amino acids with hydrocarbon side chains, as these cannot form hydrogen bonds. Of course, this assumes that the channel is expressed. A Q119C mutation might be expected to produce a constitutively open channel that could be closed by adding Cd^{2+}; the Cd^{2+} would tie the –SH residues of cysteine together, if they managed to come close enough together to complex the metal ion. However, it is too speculative to pursue these suggestions further, absent data or calculations.

The ion block may occur neither at the bundle crossing nor at the location where the side chains ligate the domains, although, with the conceivable exception of the selectivity filter, it is hard to see where another such a place could be. The hypothesis makes no statement concerning the location of the K^+ block in the closed state; it is

Table 1. Distances in Angstroms between amide oxygens and nitrogens in Q119 of KcsA, using the new (1J95) coordinates

	O1	N1	O2	N2	O3	N3	O4	N4
O1	------	2.25	4.92	**3.10**	7.04	5.38	5.00	5.31
N1		-------	5.08	*3.01*	5.38	4.28	**2.99**	*3.21*
O2			-------	2.25	5.04	**2.84**	7.13	5.57
N2				-------	5.19	*3.10*	5.55	3.81
O3					-------	2.25	4.83	**2.74**
N3						-------	5.22	*3.05*
O4							--------	2.18
N4								--------

Note: distances shown as **bold** are distances between oxygens and nitrogens in the next glutamine; italics show N – N distances. These are all at distances that form normal hydrogen bonds. The system as a whole forms a fairly good square with side about 5.0 A for the oxygens, and about 3.1 A (also hydrogen bonding distance) for the nitrogens.

Table 2. Shift in proton between two methylamines as field changes

N-N distance (A)	Field (10^9 Vm^{-1})	P(side 1)	P(side 2)	Δfield (10^9 Vm^{-1})	P(side 1)	P(side 2)
3.6	.757045	.9990	.00089	.00005	.0013	.9986
3.2	.77933	.8795	.1205	.00005	.1018	.8982
3.2	.77933	.8795	.1205	.0009	.00015	.9998
3.2	.77933	.8795	.1205	.005	8×10^{-6}	>.9999

Note: Δfield is the change in the field on the left to obtain the probability of placement of the proton noted in the last two columns. P(side 1), P(side 2) are the probabilities of finding the proton on that side (i.e., in the potential well belonging to that N atom). Note that of the three 3.2 A results all are at fields less than the 10^7 Vm^{-1} total membrane field, the middle value less than 10% of this. Results are from Yin and Green (68).

concerned only with the gating mechanism. If the K^+ can move through the channel after a conformational change separates the domains, it makes no difference, from the point of view of gating, what the reason is that K^+ cannot move through before the conformational change. Gating only requires that the conformational change must be enabled.

One other point should be mentioned here. We have already described the deuterium substitution results of Schauf, and of Alicata *et al.* On our model, they would correspond to the difference in the breaking of the hydrogen bonding network, or SSHB, that holds the channel closed. This is the last step in gating, and it depends on the provision of a proton from the channel (or for the bacterial channel, from the intracellular medium). The remainder of the model describes how a voltage gated channel could accomplish this.

4.2. Proton tunneling in the presence of a field

Yin and Green (68) performed a set of ab initio calculations on the transfer of a proton between two methylamines in the presence of a large electric field. The entire system thus had a +1 charge. As no other molecules were present, and the dielectric constant was taken as unity, the calculation could be thought of as in the gas phase, or in vacuum. However, the essential features of the proton transfer were preserved. To demonstrate the plausibility of the transfer, it is adequate to use the simplified system, which was the most that could be managed with the available computer resources.

If the two methylamines were allowed to optimize their positions, the result was a single potential well for the proton, with a N – N distance of approximately 2.7 A. In the calculation, the N – N distance was frozen at 3.2 A, or else at 3.6 A. At each distance, a potential energy surface was calculated for the proton, in three dimensions. From this, the Schrodinger equation was solved numerically, using a Fourier Grid Hamiltonian method (69) in three dimensions. The method was modified to take into account the fact that the wave function did not separate in the three dimensions, but included the correlations between the dimensions. With a field of 7.87×10^8 V m^{-1}, the proton density (from the wave function squared) was >0.99 on one nitrogen. When the field was changed by approximately 5×10^4 V m^{-1}, it switched to >0.99 on the other nitrogen. The switch was sharper at a N – N separation of 3.6 A than at 3.2 A, as would be expected. Specific results of the calculation (68) are given in Table 2. The sudden and thorough switch is consistent with tunneling of the proton. The most obvious question is whether it is plausible that so high a field, almost 10^9 V m^{-1}, should exist within the channel. There is some evidence that high fields are reasonable, as has already been discussed in the context of alignment of water in the channel. Both experimental and theoretical studies support the conclusion, showing that a high field in a channel, appreciably greater than that provided by the trans-membrane potential, is easily possible. More to the point, Asamoah et al ((40)) have shown that there is a minimum of 10^8 V m^{-1} field at a crucial arginine of the S4 segment of Shaker, by the use of a fluorescence spectral shift. This is at one end of the S4 segment, thus in a logical location for the tunneling event. It is at least possible, even likely, that the necessary field has been experimentally shown to exist in the correct location.

The existence of this high field is one principal question that had to be answered in deciding that transfer of a proton by tunneling in a channel is possible. The other major problem is the control of the tunneling event. This must be possible with the field available from the

membrane potential. Based on the calculation, the process can occur under voltage control, with a tiny change in field sufficient to trigger it. If the field change is 5 x 104 V m-1, which corresponds to about 0.35 mV across the membrane (compare the 70 mV resting potential), then the threshold for tunneling can be extremely sharp. As the calculation was done for conditions rather different than those that exist in the ion channel (the tunneling calculation was done in the gas phase, with no temperature -- hence, effectively zero temperature, and it was done for simple aliphatic amines), it is important to notice that there is about two orders of magnitude increase in the control potential possible within the range of potential available at the membrane; in fact the original calculation was not pushed to find the lowest possible potential change that would control the transfer.

The transfer of the proton is expected to be qualitatively similar with more complex systems, although external groups will affect the local field and bonded groups will affect the energy levels of the proton wells. This still allows for the possibility that tunneling might not occur, if the voltage range is quantitatively unavailable, the barrier is too high for tunneling, or if the position of the relevant amines is too close, so that only one well exists. The latter is unlikely, as the relevant amines are in fact guanidinium side chains of arginines. The spacing is far enough apart on S4 that spacing close enough to have only one well seems improbable. Neither of the other two possibilities can be immediately ruled out, absent a structure and a complete calculation. However, given the experimental fact that the initial step in gating is extremely fast and is a very small current, within the range of the magnitude expected for a single charge step part way through the membrane, it is extremely likely that tunneling is the first step in gating. The experiment shows almost the exact signature to be expected for tunneling. The fact that a field of the necessary magnitude occurs in a likely location helps understand how tunneling could occur.

Where in the 70 mV range of depolarization the tunneling transition comes is not so clear; the transition may occur over a range of values as channels are depolarized. Local fluctuations in potential will cause different channels to be locally at different potentials. However, it is only necessary for the channel to open within a reasonable range of potentials, so that the small difference in potential needed to trigger gating is not what governs the channel; the set point (the location, in voltage, at which the threshold occurs) of the channel, based on local fluctuations, may vary over tens of millivolts. Experimentally, the gating current and the open probability both follow sigmoidal curves; this is usually interpreted from the point of view of a Boltzmann (thermal activation) curve. However, the same form is preserved by assuming a threshold, with a Gaussian distribution of threshold potentials(70). Even if all channels were identical, local potentials prevent them from behaving as such. The nerve impulse drives the channels through the range that allows them to gate. At some point in this range, all channels should pass the threshold for tunneling, and begin to gate.

The time required for the process is presumably much less than the 2 μs that has been measured so far. Switching to deuterium would slow the process, but not so much as to affect the overall rate of gating, so that no conclusions can be drawn from the deuterium substitution experiments in the literature with respect to this part of the model.

4.3. Proton transfer along S4

4.3.1.Calculations of a potential energy surface

We have already mentioned that experiments of Starace and Bezanilla (8-10) demonstrated that protons could be transferred along S4 if the last two arginines were mutated to histidines. As the gating current is a capacitative current and thus must not completely cross the membrane, it is to be expected that there would be an actual proton current completely across S4 only if S4 were altered. The gating current itself is consistent with the existence of a proton current that is blocked before it completely crosses the membrane. It appears that at least two of the arginine residues in the S4 segment of the Shaker channel must be partially uncharged for the proton to move. This requires a substantial pK shift. Such pK shifts are possible, and are found in certain systems.

Other explanations of gating assume that S4 keeps its arginines all charged, and moves as either a rigid body, or makes some more complex conformational change, carrying along the charges and thus creating the gating current by a translation of the S4 charges. Presumably this movement would pull the channel open in some manner. However, it is not likely that the channel makes large scale movements (see discussion, section 2.4), and the S4 seems rather well connected to the remainder of the outer segments (S1, S2, S3), for example by salt bridges to S3 and S2 (14). There are other difficulties; for example, the S3-S4 linker can be truncated, in fact effectively removed, without preventing gating, merely slowing it (12,13,71). Therefore, it seems appropriate to look for the gating current from a source that does not require the S4 segments to make any significant motion. Proton motion has this property.

We have begun quantum mechanical calculations on proton transport between arginine residues, at about the distances present in S4. As there is no S4 structure known, we have tried a series of plausible distances between arginines. Because of the computer resources needed for the computation, the arginines have been truncated to their side chains, guanidinium groups. The computation is then carried out as a quantum mechanical optimization, with water added (two to four molecules) with the proton positioned at several locations (generally about 30) between two of the atoms (in this case, two nitrogens of the guanidinium groups). Choosing a range of proton positions and angles with respect to the guanidinium nitrogens, one gets a potential energy surface (PES) that can be filled in by interpolation. From this, a minimum energy path can be estimated, along with the presence or absence of a barrier.

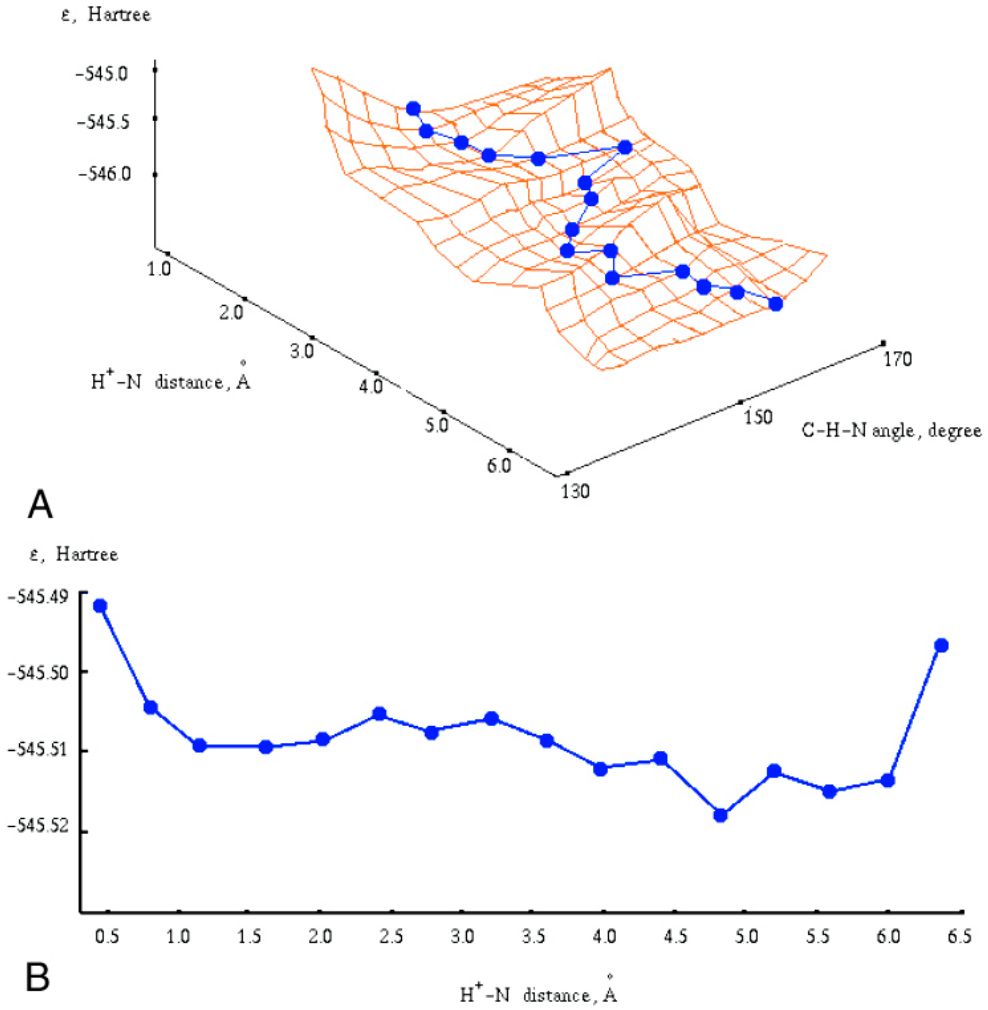

Figure 3. An example of the proton transfer calculation for two guanidiniums: In this case, the guanidinium nitrogens are separated by approximately 6.3 A, and three water molecules are present. The proton would follow a path near the waters as it passed from one nitrogen to the other. A. Potential energy surface: The energy is given in Hartrees. The minimum path is given for the energy as a function of the N – H – N angle and distance from the nitrogen. C –C distance and N – N distance are fixed. The three water molecules are allowed to rotate.). The other two nitrogens, and the hydrogens and oxygens, are not fixed, and are reoptimized as the proton is put into a different position. B. The minimum energy path as a function of distance, showing the depth of the minima and the distance of the proton with respect to the fixed atoms at each step. The energy maximum between the minima at 1.2 to 1.5, and 4.8 A, from the first N atom, is approximately 0.005 H, or about 5 kT, above the minimum, traveling from left to right. This is a reasonable height for the proton to transfer between the two wells. However, as it takes no account of surroundings beyond the three water molecules, it cannot be considered a quantitative determination of a barrier that would be found in an actual ion channel. The separation of the two wells is about 3.8 A. The water plays a key role along the pathway, as calculations without the water produce high barriers.

The calculations were done using the B3LYP density functional method (72) in Gaussian98 (73), with 6-311G** basis set. The computations were also done more inexpensively using the PM3 semiempirical method, but that proved to be inaccurate in the regions of the path where the proton was fairly close to the water molecules. Therefore only the B3LYP/6-311G** results are considered here. We can summarize the results by stating that there are several configurations of guanidinium groups that allow the proton to transfer without significant barriers (74). A more recent example of a PES of this type, and the accompanying path for the proton, is shown in Figure 3. The water molecules, which were allowed to optimize their orientation, rotated when the proton passed, in a manner that might have been guessed in advance: the water turned its oxygen in the direction of the proton. Further work is needed to determine whether the PES for a set of three guanidiniums, or even two plus a more extended structure, would also show proton transfer as easily. From the work done so far, the transfer is plausible, and, pending a

structure to work from, the proton transfer hypothesis for the gating current is also plausible. Calculation of the three guanidinium case, and the addition of the protein backbone at least adding the remainder of the arginine) should help define the calculation for a more realistic system. The entire gating current amounts to about 12 charges crossing the membrane field, or, given the four-fold symmetry, about 3 three per domain. In some cases, the number could be as high as four. The calculation will therefore also have to be done with more than one proton moving. While it is not difficult to imagine the source of the protons, the sink must be part way into the channel, and a structure is needed to see whether there are basic residues near the upper portion of the S4 segments. They need not actually be on S4 itself, but only close enough that they could accept the protons until it was time for the channel to return to the closed (not inactivated) state. There is evidence that the selectivity filter is sometimes involved in gating (more in some channels than others). However, it is hard to see where the selectivity filter could hold charges. They could not be held in the water below (intracellularly to) the filter, as the charge would be much too large, and the field impossibly high. It is more likely that they would be held by residues in S1 to S3, near the extracellular side, but a structure would be needed to see where.

4.3.2. Treatment as a ferroelectric

The structure of the S4 segment suggests that of a small ferroelectric. Ferroelectrics, like ferromagnets, consist of particles cooperatively aligned, but by electric dipoles rather than magnetic spin dipoles. There is a consequent macroscopic dipole. At a high enough temperature (the Curie-Weiss temperature (75)), it becomes impossible to maintain the alignment and the ferromagnet becomes paramagnetic, or the ferroelectric becomes paraelectric. Bystrov, Leuchtag, and coworkers (76-79) have suggested that there is an analogy between channel gating and macroscopic ferroelectrics, and that some experimental evidence could be interpreted in support of this idea. Whether the S4 segment constitutes a ferroelectric has not been demonstrated, but the following properties make it plausible to test this view of the problem:

1) There exists a class of known ferroelectrics for which the dipole consists of protons asymmetrically placed in hydrogen bonds forming a crystal, such as potassium hydrogen phosphate or triglycinesulphate (75). In addition, ferroelectric liquid crystals composed of amino acids have been reported (80); recall also the bacteriorhodopsin case mentioned earlier (54).

2) If the S4 segments each have a proton placed between two guanidinium groups, it would constitute so small a ferroelectric that it could be only formally described by a phase transition if it went from ordered (ferroelectric) to disordered (paraelectric). If all four S4 domains are even weakly coupled, however, it may be more reasonable to think of the system as ferroelectric.

3) In order to be a ferroelectric, the coupling between the protons and protein would have to produce a two-minimum potential energy surface for the protons, thus making the type of transition found in macroscopic ferroelectrics possible in S4 segments also (79,81). The calculations described in the preceding section, on proton transport, will also test this idea. Some cases do seem to have the correct form, although much further work will be required to determine the validity of the analogy.

If the system is thought of as ferroelectric, and the motion of the protons is cooperative, one step of gating could be treated as the kinetics of a phase transition.

5. CONCLUSIONS

There are a large number of ion channels that either gate with protons, or whose gating is influenced by pH. The KcsA channel has a structure resembling that of the inner part of the standard form of the K^+ channel, without the segments responsible for voltage gating. While the detailed conformations, and molecular hinges on which the inner segments rotate, differ, the overall mechanism seems sufficiently similar to allow it to be used as a model for one step of gating, that is, for the step in which the channel is held together by a strong short hydrogen bond. As far as can be determined the bacterial channel, KcsA, gets a spare proton from a source external to the channel. However, the eukaryotic channels may be able to deliver their own protons. This requires the channel to trigger a proton cascade in response to membrane depolarization, and then have the proton cascade along the S4 segment deliver the proton to a SSHB in a position to weaken the bond, and thus open the channel. Each of these steps seems plausible based on experimental and theoretical considerations, but more work is needed to know whether the model is definitely correct or not.

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