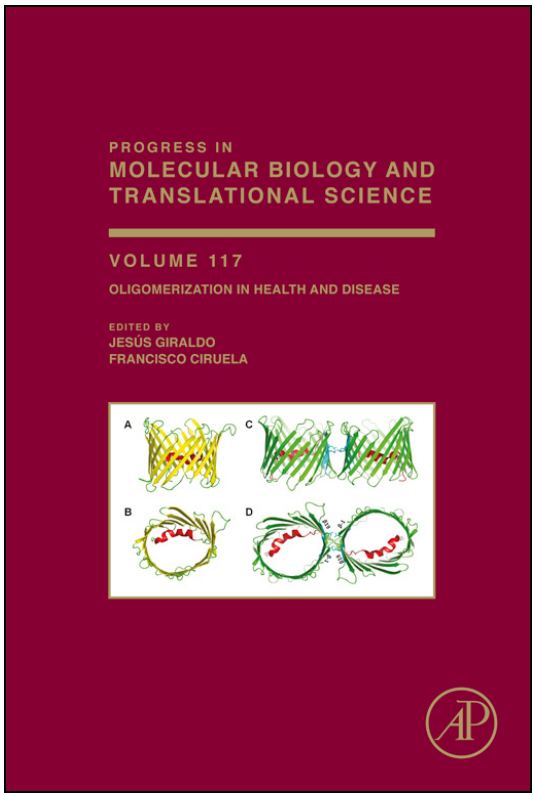

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CHAPTER TWELVE

Consequences of Dimerization of the Voltage-Gated Proton Channel

Susan M.E. Smith[*], **Thomas E. DeCoursey**[†]
[*]Department of Pathology and Laboratory Medicine, Emory School of Medicine, Atlanta Georgia, USA
[†]Department of Molecular Biophysics and Physiology, Rush University, Chicago Illinois, USA

Contents

Abstract

The human voltage-gated proton channel, hH_V1, appears to exist mainly as a dimer. Teleologically, this is puzzling because each protomer retains the main properties that characterize this protein: proton conduction that is regulated by conformational (channel opening and closing) changes that occur in response to both voltage and pH. The H_V1 dimer is mainly linked by C-terminal coiled-coil interactions. Several types of mutations produce monomeric constructs that open approximately five times faster than the wild-type dimeric channel but with weaker voltage dependence. Intriguingly, the quintessential function of the H_V1 dimer, opening to allow H^+ conduction, occurs cooperatively. Both protomers undergo a conformational change, but both must undergo this

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transition before either can conduct. The teleological purpose of dimerization may be to steepen the voltage dependence of channel opening, at least in phagocytes. In other cells, the purpose is not understood. Finally, several single-celled species have H_V that are likely monomeric.

1. INTRODUCTION

Ion channels comprise a large family of membrane proteins that regulate the passage of ions, usually one particular species of ion, across cell or organelle membranes. Most ion channels are multimeric, but dimeric channels do occur, albeit infrequently. The voltage-gated proton channel (H_V1) gene (*HVCN1*) was identified in 2006,[1,2] and its dimeric nature was discovered shortly thereafter.[3–5] The main focus of this review is to survey the consequences of dimerization for the molecular and biological functions of these channels. On the molecular scale, proton channels open and close, conduct only protons when they are open, and characteristically are regulated closely by the pH gradient.[6] Voltage-gated proton channels in humans (hH_V1) have diverse functions in a variety of cells. They participate in pathogen killing by phagocytes,[7–11] histamine secretion by basophils,[12] surface pH regulation by airway epithelia,[13] capacitation and motility of sperm prior to fertilization,[14,15] B lymphocyte signaling,[16] and may exacerbate breast cancer metastasis[17] and brain damage in ischemic stroke.[18]

2. DIMERIZATION OF MEMBRANE PROTEINS

A number of ion channels function only when assembled into the multimer that forms the ion-conducting structure. For example, voltage-gated K^+ channels are tetramers that use two transmembrane (TM) helices (S5–S6) from each of the four subunits to produce a single, central K^+ selective pore (Fig. 12.1). In a few ion channels, including H_V1, individual monomeric units appear to be fully functional, yet the native channel assembles as a dimer or oligomer with properties distinct from the monomer. The high frequency of membrane protein homodimers (and higher order homo-oligomers) argues that there is a strong evolutionary advantage for this kind of quaternary structure. Commonly accepted "reasons" for dimerization of membrane (and soluble) proteins include increased stability, increased specificity of interaction with regulatory proteins, regulation or modulation of activity, and acquisition of cooperativity. H_V1 activity is modulated by at

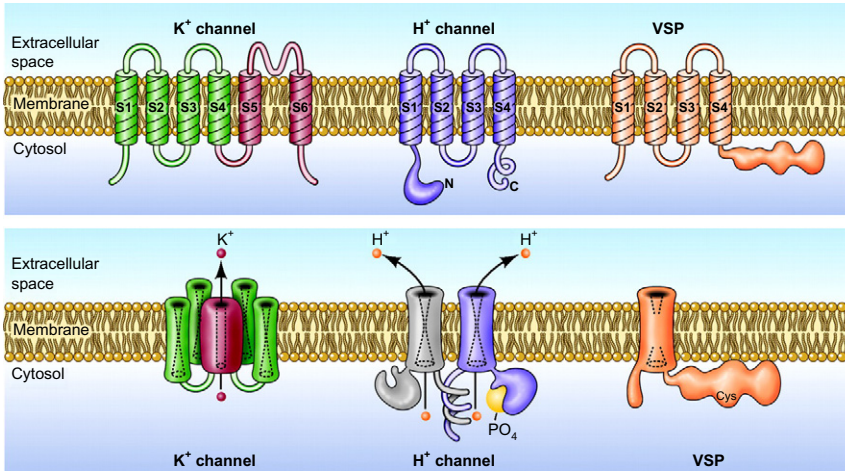


Figure 12.1 Architecture of three classes of VSD containing molecules. The top row shows the monomeric protein; the lower row shows the final assembled protein. The K^+ channel assembles as a tetramer,[19,20] with four separate VSD elements connecting to a single central pore (the pathway taken by K^+ as it permeates). The voltage-gated H^+ channel in many species, including mammals, assembles as a dimer,[3–5] although when constrained to exist in monomeric form, it retains its key properties of proton-specific conduction, voltage gating (opening upon depolarization of the membrane potential), and ΔpH dependence that strongly regulates the opening of the channel on the basis of pH. The VSP, a voltage sensitive phosphatase, is thought to exist and function as a monomer.[21] *Reprinted from Ref. 9, copyright 2010.*

least one protein kinase, although no evidence exists one way or the other for differential specificity of the kinase for monomeric or dimeric H_V1. H_V1 dimers do act cooperatively, which has implications for physiological interplay with NADPH oxidase function in human neutrophils. Although not definitely established, dimer assembly does not appear to be involved in enhanced gating, the best characterized functional modulation of H_V1.

3. EVIDENCE THAT PROTON CHANNELS EXIST AS DIMERS

A variety of data supports the dimeric nature of the native H_V1 in humans, mice, and *Ciona intestinalis*.[3–5,22–26] The clearest and the most direct evidence that hH_V1 are dimers was provided by Tombola *et al.*,[5] who attached green fluorescent protein (GFP) to the channel molecule, and then observed photobleaching of individual channel molecules. As illustrated in Fig. 12.2, the fluorescence intensity of most channels decayed in two

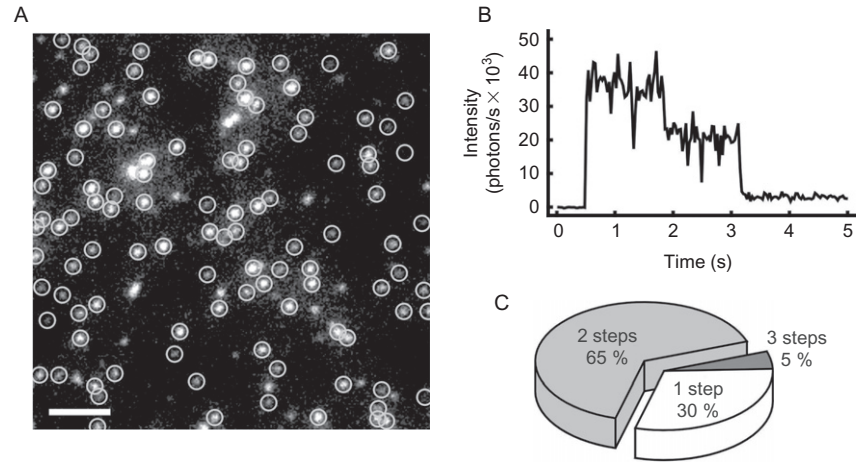


Figure 12.2 Evidence that the human proton channel, hH_V1, is a dimer. (A) shows GFP (green fluorescent protein)-tagged hH_V1 channels visualized under fluorescence microscopy. Circled spots were followed over time, as illustrated in (B), where the fluorescence intensity of one spot can be seen to decay in two distinct steps. The pie chart in (C) shows the frequency that tagged channels decayed with the indicated number of steps. *Reprinted from Ref. 5, copyright 2008, with permission from Elsevier.*

discrete steps. Additional evidence that hH_V1 is a dimer, and that each protomer has a separate conduction pathway, was provided by tandem dimers including an introduced Cys at a location that enables block of the channel by a methanethiosulfonate reagent. Wild-type (WT)–WT dimers were not blocked, WT–Cys dimers were half blocked, and Cys–Cys dimers were completely blocked.[5] Some impairment of dimerization was seen when the N-terminus was disrupted or replaced, but dimerization was completely prevented by C-terminus substitution.[5]

Koch *et al.*[3] demonstrated the multimeric nature of the mouse H_V1, mH_V1 (which they called mVSOP), by coimmunoprecipitation of the channel labeled with two different tags. They further used fluorescence resonance energy transfer (FRET) to determine the distance between tagged residues in an extracellular loop of *C. intestinalis* H_V1 (CiH_V1 or CiVSOP) protomers to be 42 Å, also consistent with their being multimers. They showed that the preferred multimeric configuration was a dimer by creating a linked heterodimer including a single introduced Cys residue. Lack of FRET in this construct showed that only two subunits are involved. Gonzalez *et al.*[22] demonstrated FRET in full-length CiH_V1, which disappeared in the N- and C-terminal truncated channel, showing that this construct was monomeric.

Another kind of evidence is provided by the cross-linker disuccinimidyl suberate (DSS), which produced a distinct band at the dimer position on western blots.[3,4,26] In one study, there were weak bands at positions corresponding to higher oligomers,[4] in the other not.[3] The higher order oligomer bands could be nonspecific artifacts, as suggested by Lee and coauthors, but at least some could also represent natural, weaker interactions. Overexpression, cross-linking, and Western blotting all suffer from artifacts, so results must be interpreted with caution; still, these two studies used different H_V1 proteins, cell lines, and methods, greatly increasing the probability that they represent normal interactions. Further increase in confidence is provided by the demonstration of the dimeric nature of native hH_V1 *in situ* in human neutrophils[26] using western blots. Faint bands at the dimer level, which were greatly enhanced by DSS treatment, were seen in lysates from human neutrophils, eosinophils, and monocytes.

3.1. Not all H_V1 are likely to be dimers

Direct evidence exists for the dimeric tendencies of H_V1 in humans, mice, and *C. intestinalis*. However, because the most important determinant of dimeric status is C-terminal coiled-coil interaction,[3–5,22–26] H_V1 lacking this property may exist as monomers. Of confirmed or predicted H_V1, those in the dinoflagellate *Karlodinium veneficum*[27] and in the diatoms *Phaeodactylum tricornutum*[28] and *Thalassiosira*, lack predicted coiled-coil domains and are presumed to be monomeric. H_V1 in coccolithophores, which are also single-celled eukaryotes, contain predicted C-terminal coiled-coil domains, which precludes any generalization regarding distinctions between oligomerization in multicellular and unicellular organisms. At present, there is no evidence that any accessory proteins are involved in or required for dimerization. The proton conduction that is the biological function of hH_V1 can be demonstrated when the purified protein is incorporated into membrane vesicles.[29] Further characterization of the physiological roles of H_V1 in various organisms will be needed to understand any distinction in the roles of dimeric versus monomeric channels.

4. COMPARISON OF THE PROPERTIES OF MONOMERIC AND DIMERIC CONSTRUCTS OF H_V1

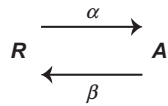
Exploring the differences in behavior between monomeric and dimeric constructs of H_V1 is of intrinsic interest but may also provide clues to the evolutionary "purpose" of dimerization. A word of caution must be

proffered before accepting the following comparison. Because (most) H_V1 normally exist as dimers, unnatural, and invariably extreme measures must be adopted to force it into monomeric status. For example, truncation of the N- and C-termini must be considered drastic measures. These manipulations themselves may have consequences other than simply producing monomeric channels.

4.1. What does "cooperative gating" mean for ion channels?

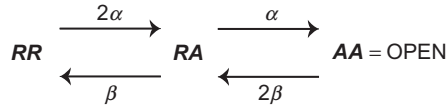
Cooperative gating of H_V1 has been discussed with the idea that the two protomers do not function independently. In order to understand the evidence that exists for cooperative gating in H_V1, we will first consider the idea of cooperativity as it relates to ion channels. Cooperative binding of substrates to proteins that have multiple-binding sites is a straightforward concept. A well-known example of (positive) cooperativity is that the first O_2 to bind the tetrameric hemoglobin molecule increases the O_2 affinity of the three remaining binding sites. A classic description of cooperativity in ion channels was provided by Hodgkin and Huxley.

Hodgkin and Huxley observed that both Na^+ and K^+ currents in squid axons activated (turned on) with a delay,[30] which they could explain by postulating multiple identical voltage-sensing elements, all of which must move before the conductance appears. A mathematical representation of this idea is to assume identical conformational changes in each element, each a simple first-order transition between a resting state (***R***) and an activated state (***A***) (Scheme 12.1):



Scheme 12.1 Two state model.

where the forward and backward rate constants are α and β. On average, the movement of one element (protomer) after a step change of voltage follows an exponential time course, with time constant, $\tau_{act} = 1/(\alpha + \beta)$. Both rate constants are typically voltage-dependent, and the steady state probability of ***A***, the activated state, at a given voltage is $\alpha/(\alpha + \beta)$. If we assume that the channel can conduct only after both voltage-sensor domains (VSDs) in a dimeric channel reach state ***A***, then the opening process can be shown as in Scheme 12.2.



Scheme 12.2 Hodgkin-Huxley model.

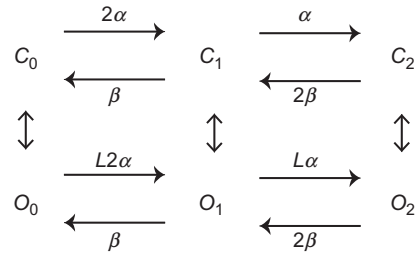
Scheme 12.2 describes the Hodgkin–Huxley (HH) model for a two component channel. In this scheme, the current will turn on with a sigmoid time course, and upon repolarization, will turn off with an exponential time course, because once either protomer moves to its ***R*** state, conductance ends.

Turning to H_V1, we have discussed evidence for dimers consisting of two identical protomers, each with a distinct conduction pathway and some means of responding to membrane potential changes. In the simplest case, movement of charged groups within the TM helices of the protein would produce a conformational change that directly results in conductance ("channel opening"). In this case, as soon as the first protomer "opened" there would be current. Nonindependence in this scenario could reflect interactions between protomers, such that opening of one facilitates (or inhibits) the opening of the other, exhibiting positive (or negative) cooperativity, in a sense similar to O_2 binding in hemoglobin.

A different class of nonindependence would arise if interactions within the H_V1 dimer resulted in conductance occurring only after both protomers "open." In the sense that conductance requires both members of the dimer to perform, this can be considered cooperative, but this phenomenon can also occur if each protomer moves independently of the other in response to voltage changes. The cooperativity in this example results not from interactions during the initial response to voltage but during a subsequent step during which the conductance appears.[31] This is the kind of cooperativity modeled by Hodgkin and Huxley.[32]

A slightly more complicated model was proposed by Gonzalez *et al.*[22] They postulated an "allosteric" mechanism, in which the $\boldsymbol{R} \rightarrow \boldsymbol{A}$ transition does not immediately result in conductance, even in a monomer, but instead an additional transition must occur: $\boldsymbol{R} \rightarrow \boldsymbol{A} \rightarrow \boldsymbol{O}$, where $\boldsymbol{O}$ is the open (conducting) state. Here, the $\boldsymbol{R} \rightarrow \boldsymbol{A}$ transition (horizontal steps) reflects the movement of the fourth TM helix (S4) in response to voltage into an activated configuration, and the $\boldsymbol{A} \rightarrow \boldsymbol{O}$ transition (vertical steps) reflects a conformational change that results in conductance (channel opening). For the H_V1 dimer, a simplified version of the allosteric model is shown in Scheme 12.3.

Here, the $\boldsymbol{C}_n$ states are closed (nonconducting), and the $\boldsymbol{O}_n$ states are open (conducting). The allosteric factor L (which also appears in the vertical



Scheme 12.3 Allosteric model.

transitions, but is not shown here) determines how much interaction exists between protomers. Aside from additional complexity, the allosteric model differs in one significant respect from the HH model. This point requires a preliminary discussion of gating charge.

4.2. What is gating charge and how is it measured?

A voltage-dependent ion channel opens in response to a change in membrane potential. This occurs because there are net charges within the membrane spanning regions of the protein that move in response to voltage changes. For example, the S4 region of most voltage-gated ion channels contains several positively charged Arg (or Lys) residues, and when the membrane is depolarized (by making the inside of the cell more positive), these positive charges are pushed outward. "Gating charge" refers to the number of elementary charges (e_0) in each channel molecule that must be transferred from one side of the membrane to the other during the opening process. If one charge travels across only half the membrane potential field, this would count as 0.5 e_0. One additional complication is that the membrane potential does not change uniformly, as was assumed by Goldman in his "constant field theory,"[33] but is focused at the narrowest part of the pore.[34–37] A standard method for estimating the effective gating charge is called the "limiting slope" method. The steady state conductance (which is assumed to reflect approximately the open probability of the channel, P_{open}) is plotted semilogarithmically against voltage. Almers showed that the slope at the most negative voltage range that can be detected is a good estimate of the effective gating charge for any gating model in which the channel traverses an arbitrary number of closed states to arrive at a single open state.[38] More detailed analysis of which kinds of models follow or deviate from this guideline is provided by Sigg and Bezanilla.[39] For Na^{+}[40] and *Shaker* K^{+} channels, the total effective gating charge estimated by this method is 12–14 e_0.[41–44] To a rough approximation, this estimate corresponds well

with the physical manifestation expected if four Arg residues from each of the four VSDs of the tetrameric channel, crossed most of the membrane electrical field. Returning to the models described earlier, the limiting slope method applies to the HH model (Scheme 12.2), but not strictly to the allosteric model (Scheme 12.3). The reason for this is that the allosteric model permits (a) channel opening before all the VSDs have moved and (b) transitions between open states. If these transitions involve movement of gating charge, then the limiting slope will underestimate the true gating charge.[39] An excellent example of this phenomenon is the BK channel, whose g–V relationship at moderately negative voltages first becomes steeper, but at larger negative voltages becomes much shallower.[45] An allosteric model described the gating of this channel well, and explained the anomalous limiting slope data, because channels can open before all the voltage sensors have moved and also because charge movement can occur during transitions between open states.[46] The error introduced by using the limiting slope approach depends on the degree of allosteric interaction; at one extreme, the allosteric model degenerates into a linear model (in which all channels follow the $C_0 \rightarrow C_1 \rightarrow C_2 \rightarrow O_2$ pathway in Scheme 12.3), in which case the limiting slope gives the correct value.

If we consider the K^+ channel in Fig. 12.1, the physical embodiment of the HH model (with movement of all four VSDs required before opening occurs) is clear. There are four identical VSDs, each of which must undergo a conformational change before the central pore is opened.[30,47,48] For H_V1, the physical interpretation is less obvious, because there are two separate pores.

4.3. Evidence that gating of the two protomers in H_V1 is "cooperative" (not independent)

Evidence already discussed shows that each protomer in H_V1 contains a separate conduction pathway. It has become clear, however, that the gating (opening and closing in response to voltage changes) of the two protomers does not occur independently. One line of evidence comes from coexpressing roughly equal amounts of WT hH_V1 and a mutant, E153C, that has the property that its proton conductance–voltage (g_H–V) relationship is shifted—50 mV compared with WT. Independent gating of the protomers should result in a component of negatively shifted channels. On the other hand, if both protomers must undergo a conformational change that precedes conduction of either, channels should open at the more restrictive (more positive) regime. When the experiment was performed, the resulting g_H–V relationship practically superimposed on that of the

WT channel, which has the more positive voltage requirement. Linked heterodimers shared the nearly WT g_H–V relationship seen in the coexpression studies. A puzzling feature of these results was the lack of a shoulder on the g_H–V relationship in the coexpression studies, which would be expected from the fraction of double mutant dimers.

Gonzalez *et al.*[22] also produced elegant evidence for cooperative gating (Fig. 12.3C). They labeled the outer end of the S4 domain with a fluorophore and used voltage-clamp fluorometry to observe S4 movement simultaneously with proton currents during voltage pulses. The fluorescence signal reflecting S4 movement (red) increased exponentially, whereas the proton current (black) increased with a sigmoid time course. In the traditional Hodgkin–Huxley[32] formalism of Scheme 12.2, the time course of the fluorescence signal raised to the second power (green) will predict the time course of the current if the two protomers gate independently and if both must undergo a conformational change before either conducts. It is a happy event when data cooperate so nicely with theoretical expectations!

Further evidence supporting this form of cooperative gating emerged when the Larsson group determined the effective gating charge of hH_V1. WT H_V1 channels have an effective gating charge of ~6 e_0 in rat[49,50] and human.[51] In CiH_V1, the WT channel had 5.9 e_0 but the monomeric construct had 2.7 e_0.[22] This result strongly supports the idea that both protomers must move before either can conduct. A recent study of the mouse Hv1, mH_V1, produced identical phenomenology: the dimeric channel had twice the effective gating charge as seen in monomeric constructs.[25] In this species, the gating charge in WT channels was 4 e_0, with 2 e_0 seen in monomeric constructs.

4.4. Activation kinetics differs between monomer and dimer

Koch *et al.*[3] produced monomeric mH_V1 (mouse) channels by truncating the C-terminus and also by truncating both C- and N-termini. The C-truncated channel (ΔC) opened 2.5 times faster, and the doubly truncated channel (ΔNΔC) opened five times faster. If we interpret the double truncation as more efficiently producing monomeric channels (i.e., that some dimers remain even with C-truncation), then the monomer opens five times faster. In a later study, C-truncation of mH_V1 accelerated channel activation, τ_{act}, by ~sixfold (Fujiwara *et al.*).[25]

Tombola *et al.*[5] produced monomeric chimerae by attaching the C-terminus alone or both the N- and C-termini from the CiVSP, a phosphatase from *C. intestinalis* thought to exist as a monomer,[21] onto hH_V1. A minor

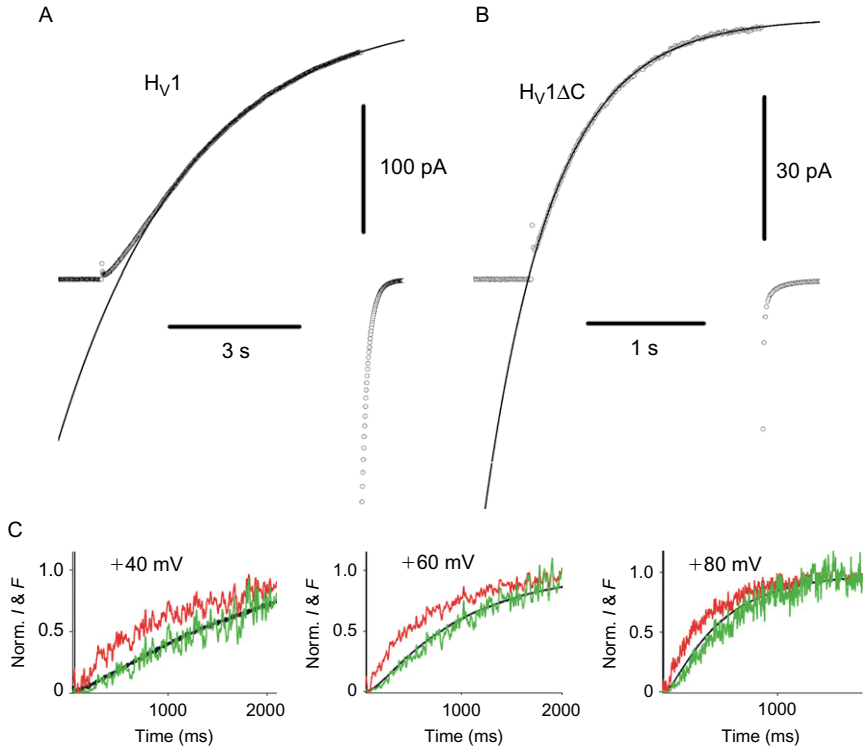


Figure 12.3 Gating kinetics of monomeric and dimeric hH_V1 and CiH_V1. The sigmoid activation of WT dimeric hH_V1 channels (A) is contrasted with the more rapid and exponential turn-on of C-truncated channels (B), presumed to be monomeric. The curves are single exponential fits. In (C), the kinetics of current activation of CiH_V1 at three voltages (black) is compared with the normalized fluorescence signal (red) emitted by a fluorophore tag attached to the top of the S4 domain. When the fluorescence signal is squared (see Scheme 12.2), it practically superimposes onto the current, consistent with a Hodgkin–Huxley-type gating mechanism in which both protomers must "activate" before either can conduct. *Figures A and B: Reprinted with permission from Ref. 24, copyright 2010. Figure C: Reprinted by permission from Ref. 22, copyright 2010.*

effect of swapping the N-terminus alone was also observed. The chimera with both N- and C-termini from CiVSP grafted onto the hH_V1 TM domains activated substantially more rapidly, although no quantitative results were provided. The g_H–V relationship for this monomeric construct was 30% less steep and its midpoint voltage about 10 mV more positive.

Musset *et al.*[24] produced presumed monomeric hH_V1 by C-truncation ($hH_V1\Delta C$). Although independent confirmation that C-truncation produces mainly monomers was not obtained, the activation kinetics was well fitted by a single exponential function (Fig. 12.3B). Activation of the

C-truncated channel was 6.6 times faster than the WT dimer, measured in inside–out membrane patches at pH_o 7.5, pH_i 7.5. The difference was less pronounced in whole-cell data at pH_o 7.0, pH_i 6.5, with the $hH_V1\Delta C$ about three times faster than WT.[52] Consistent with the report of Tombola *et al.*,[5] the g_H–V relationship was somewhat less steep in the monomeric construct, with the midpoint shifted 10–15 mV more positive than WT. The $hH_V1\Delta C$ construct not only opened more rapidly but also the time course of turn-on of current was exponential (Fig. 12.3B), in contrast with the distinctly sigmoidal time course of WT currents (Fig. 12.3A).[24] This behavior is consistent with the HH-type model discussed earlier (Scheme 12.2) in which each protomer must activate before either can conduct. In summary, gating kinetics supports the idea of cooperative gating.

A mechanism for cooperative gating of mH_V1 was proposed recently by Fujiwara *et al.*[25] A number of mutations were carried out in the C-terminal domain. The short region between the C-terminal (intracellular) end of S4 and the start of the coiled-coil region was deleted, extended, or replaced with different amino acids. The resulting mutants fell into two groups: one group (including the monomeric ΔC) opened several times more rapidly and had an effective gating charge of 2 e_0 based on limiting slope measurements, half that of 4 e_0 in the other group (including WT channels). Cooperative gating is consistent with the observation that the slower opening phenotype had twice the gating charge. Several conclusions can be drawn. In general, coiled-coil dimer assembly inhibits activation of H_V1, in the sense that the dimer opens more slowly. Second, the mere fact that the channel was dimeric did not ensure cooperative gating, because a flexible linker (GGG) inserted between S4 and the C-terminus prevented cooperativity. Similarly, a dimer comprising two C-truncated protomers failed to exhibit cooperativity. A related conclusion is that interactions occurring within the TM regions evidently are not sufficient to ensure cooperativity, because these constructs were shown to be dimeric, yet exhibited no signs of cooperativity.

The Okamura group[25] proposed that the C-termini that are known to be linked in the dimer by coiled-coil interactions[4,23] directly modulate S4 movement that results in channel opening. The C-termini are considered to be part of a rigid rod extending through the S4 helix, so that movement of S4 in one protomer would be linked mechanically via the C-termini to the second S4 domain. It is not precisely clear what degree of independence is allowed in this model. Thus, if both entire C-terminus S4 domain assemblies were completely rigid, both protomers would be expected to move

simultaneously. If the result of this concerted movement were direct activation of the conductance (opening of the channels), then the time course of current turn-on would be exponential, not sigmoid. Given that the movement of S4 observed directly by Gonzalez *et al.*[22] had an exponential time course, but the current has a sigmoid turn-on (Fig. 12.3C), one possibility is that a certain degree of flexibility in the structure allows a delay between the movement of the two S4 domains. Alternatively, there must be one or more additional steps that couple S4 movement to opening of the conduction pathway that would account for the delayed turn-on of the current. The Larsson group[22] could model their results either with the assumption (a) that the two protomers move independently and both must move before conduction occurs in either or (b) that there is a strong allosteric interaction that favors coordinated movement of both S4 domains over the movement of a single protomer. The latter model was also proposed by Tombola *et al.*[53]

Distinguishing these two classes of models may be difficult, especially when there is a sufficiently high degree of allosteric coupling, because the allosteric mechanism becomes indistinguishable from the HH model. Indirect evidence that seems to support the allosteric model comes from attempts to measure single channel current amplitude in H_V1. Unitary H^+ currents are $\sim 10^3$ smaller than those of most ion channels and consequently are very difficult to resolve directly. However, under favorable conditions (absence of other conductances, extremely high-resistance seals (up to 5 TΩ) which minimize noise,[54] maximized conductance induced by using low pH_i), Cherny *et al.*[55] were just able to resolve what appeared to be unitary currents, of $\sim$10 fA amplitude. An alternative method of measuring unitary currents involves analyzing current fluctuations resulting from stochastic opening and closing of channels.[56] Current variance was well resolved in excised membrane patches from human eosinophils and provided reproducible estimates of single channel conductance.[55] However, the values derived from current fluctuations were consistently about twofold smaller than those from direct observation. Concerted opening of both protomers would result in channel openings with a conductance double that of each monomer, while allosteric interaction would allow two separate steps. The current fluctuation results can be reconciled with the apparent double-sized unitary current events if allosteric interaction results in both protomers opening in rapid succession, so that the current appears to comprise a single step, rather than two steps. Because the unitary current amplitude was just on the borderline of detectability, caution should be exercised before putting too much weight on this kind of interpretation.

5. PROPOSALS FOR THE H_V1 DIMER INTERFACE

The first proposal[4] for the orientation of the hH_V1 dimer is shown in Fig. 12.4A. Cys residues were introduced at numerous locations, and cross-bridge formation was evaluated. Several cross bridges were detected at the extracellular end of S1 (red cylinders, extracellular is toward the top of Fig. 12.4) and in the S1–S2 linker; this was proposed to be the main interface region, in addition to the predicted coiled-coil interaction of the C-termini.[4] This dimer orientation is supported by FRET data obtained from *Ciona* Hv1[3] that indicated a distance of 42 Å between Cys^{243} on each protomer. Cys^{243} of CiHv1 is on the S2/S3 linker (connecting green and blue helices in Fig. 12.4), predicted to be far apart in a dimer connected at the S1 helices.

On the other hand, data consistent with more than one dimer interface also exist. In the Lee *et al.*[4] study, distinct crosslinks at hH_V1 position 194, also in the S2/S3 linker, were observed, indicating that this position attains close proximity a measurable fraction of the time, which would be unexpected if the only possible interface were through the S1 helices.

Additional evidence for another dimer interface comes from the use of Zn^{2+}, the most potent inhibitor of H_V channels,[57–60] which acts by preventing channel opening. Although sensitivity to Zn^{2+} has been examined almost religiously as a pathognomonic feature that must be confirmed in any newly discovered proton conductance in cells or species not

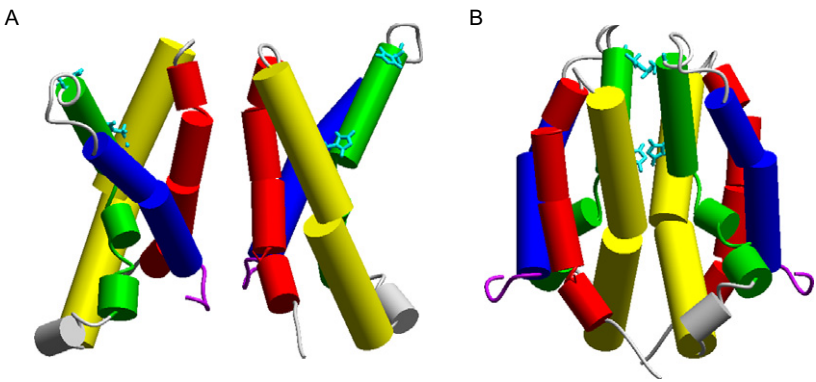


Figure 12.4 Two proposed dimer interfaces. Cross-linking studies indicate several points of attachment at the top (outer) end of S1 (red).[4] The binding of Zn^{2+} to various hH_V1 mutants with His^{140} or His^{193} (aqua) replaced by Ala suggested that high-affinity bidentate Zn^{2+} binding occurs at the dimer interface.[24] *Reprinted with permission from Ref. 24, copyright 2010.*

previously studied, Zn^{2+} sensitivity is extremely sensitive to pH.[57] The sensitivity of the channel to externally applied Zn^{2+} becomes quite weak at low pH_o, strongly suggestive of competition between H^+ and Zn^{2+} for the binding site where Zn^{2+} exerts its inhibitory effects. By mathematically modeling the competition between H^+ and Zn^{2+} at different pH_o, Cherny and DeCoursey concluded that Zn^{2+} must be multiply coordinated, and that the pK_a of the coordinating groups was roughly 6–7, suggesting that two or more His residues formed the Zn^{2+}-binding site.[57]

When the hH_V1 gene was identified, and two externally accessible His residues (His^{140} and His^{193}) were shown to account for the inhibitory effects of Zn^{2+},[1] the obvious conclusion was that these two His residues most likely coordinated Zn^{2+} at its site of action. However, in a molecular model of the open-state monomer of hH_V1, His^{140} and His^{193} were too far apart to coordinate Zn^{2+} plausibly.[24] Protein:protein docking of the model structure indicated the possibility that His pairs from different protomers could approach close enough for Zn^{2+} coordination to occur in a dimer whose interface was between the S2/S3 helices.

Several types of experiments were carried out to test the hypothesis of interprotomer Zn^{2+} coordination. The first prediction of this hypothesis is that the affinity of Zn^{2+} for the dimeric channel should be greater than for the monomer, which would lack the possibility of bidentate coordination. Indeed, C-terminally truncated constructs of both human and mouse H_V1 (presumed to be monomeric—see earlier) exhibited significantly diminished response to Zn^{2+}, consistent with the prediction. This result conflicts with a report using a different H_V1 monomer construct.[53] Because this report was based on measurements under one set of conditions at one pH at a single voltage,[53] a chance combination of separate Zn^{2+} effects may have canceled each other out. Zn^{2+} has complex pharmacological effects, including profoundly slowing activation, shifting the g_H–V relationship positively, and probably also decreasing $g_{H,max}$.[57] It is also possible that the different monomeric constructs responded differently to Zn^{2+}.

Another set of experiments involved testing mutants and tandem constructs in which alanine replaced the histidines in several combinations. Figure 12.5 illustrates the constructs tested and their relative Zn^{2+} sensitivity, using the slowing of channel opening as the parameter of interest. A similar pattern was observed when the shift of the g_H–V relationship by Zn^{2+} was the parameter evaluated. As previously shown by Ramsey *et al.*,[1] mutation of His^{140} or His^{193} alone attenuated but did not eliminate the Zn^{2+} sensitivity, demonstrating that both residues contribute to WT Zn^{2+} sensitivity. The double

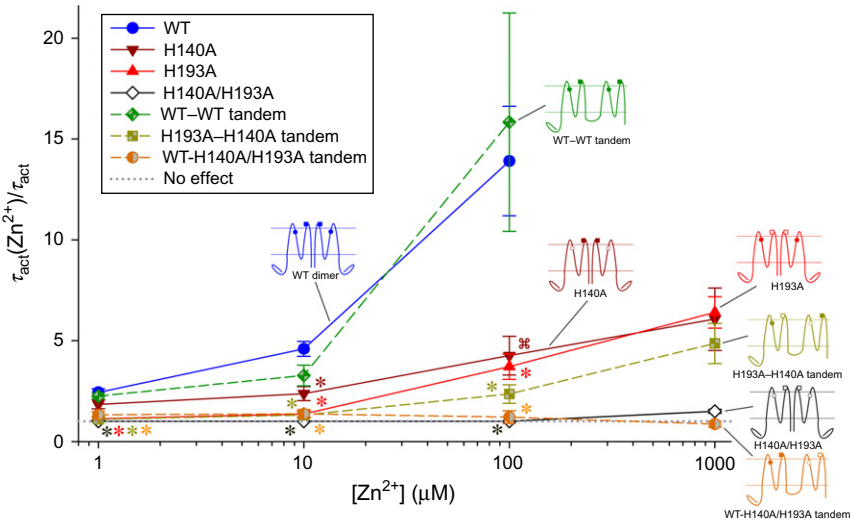


Figure 12.5 Evidence that Zn^{2+} binds with high affinity at the interface between protomers in the hH_V1 dimer. The slowing of proton current activation by Zn^{2+} is plotted for several hH_V1 constructs. The constructs listed in the inset are illustrated with His^{140} shown as a circle and His^{193} as a square. Solid symbols indicate His at the indicated position, open symbols indicate replacement with Ala. *Reprinted with permission from Ref. 24, copyright 2010.*

mutant lacked any slowing up to 1 mM Zn^{2+}, suggesting that no other groups on the channel contribute significantly. Control tandem dimers in which both protomers possessed both histidines showed Zn^{2+} sensitivity indistinguishable from WT (Fig. 12.5, blue vs. green symbols) as well as sigmoidal activation kinetics suggestive of cooperative gating. A tandem dimer in which one protomer lacked His^{140} and the other protomer lacked His^{193} ("H193A–H140A tandem") exhibited Zn^{2+} sensitivity similar to single mutants in the WT background (Fig. 12.5). A surprising result was observed for a tandem dimer in which one protomer was WT, with both His present, while the other protomer lacked both His. Just as for the double His mutant (lacking both His), Zn^{2+} did not slow opening in the tandem dimer in which both histidines were present in one protomer, while the other protomer lacked His. In summary, slowing of channel opening by Zn^{2+} was observed only when there was at least one His in each protomer. Taken together, these data indicated that Zn^{2+} is most likely coordinated between protomers, which implies a dimer interface involving S2 and S3 helices (Fig. 12.4B).

The apparent discrepancy between the two suggested dimer interfaces could be explained in at least two ways. In one, the TM domains of the two protomers, although tethered by coiled-coil interactions within the C-termini, may be able to rotate around the tether and adopt different orientations relative to each other. This possibility appears to be less likely in light of the proposed rigidity of the linker between S4 and the coiled-coil region.[25] Nevertheless, a tandem WT–WT dimer, linked with a short (6 AA) connection between the C-terminus of one monomer and the N-terminus of the second, functioned almost identically to the WT channel, with the exception of somewhat slower opening kinetics.[24,52] This result might indicate that the required C-terminal interaction can occur almost normally despite the proximity of an attached N-terminus. Another explanation posits the formation of a tetramer consisting of a dimer-of-dimers; this complex maybe stabilized by Zn^{2+} binding at an interface at the S2/S3 helices. Wherever Zn^{2+} binds, it clearly stabilizes the closed conformation, because it slows channel opening, and shifts the g_H–V relationship in the positive direction.[57]

It might be objected that the molecular model that gave rise to the hypothesis of Zn^{2+} binding between protomers is presumed to be an open-state model, whereas the general interpretation of Zn^{2+} effects presumes that Zn^{2+} binds to the closed channel. The relative positions of His^{140} and His^{193} were estimated in the predicted closed state, based on the positions of the corresponding residues in molecular models of closed $K_V1.2$ VSDs.[61] The estimated closed state positions do not differ greatly from those in the open state—in particular, they still do not appear to approach closely enough to coordinate Zn^{2+}. This is not unexpected, considering that neither His is located on the S4 helix, which is thought to be the main "moving part" of the H_V1. In any case, the results obtained do not depend on perfect accuracy of the molecular model. When the crystal structure of the closed hH_V1 molecule has been obtained, this direct evidence should resolve many questions that at present can only be addressed indirectly.

6. PHYSIOLOGICAL CONSEQUENCES OF DIMERIZATION OF H_V1

The most consistent difference between monomeric and dimeric H_V1 is in gating kinetics. However, this difference, while clear, is not dramatic. In some situations, the three- to sixfold faster monomeric opening rate

might seem to be an important distinction, but considering that among species, H_V1 activation kinetics varies over several orders of magnitude,[62] it would appear that a species with a need for quickly opening proton channels would be better served in making a channel that is intrinsically fast. H_V in snail neurons open within a few milliseconds,[63] whereas mammalian H_V1 require seconds.[64] From this perspective, fundamental kinetics would most logically be determined genetically, not by adjusting multimerization. On the other hand, if it were possible for a cell to switch its H_V1 between monomer and dimer status, then a fivefold change in gating kinetics might have important consequences. In fact, Koch *et al.*[3] initially suggested that this kind of mechanism might be responsible for the "enhanced gating mode" of proton channels in phagocytes.

6.1. Does the enhanced gating mode in phagocytes reflect dimer-to-monomer conversion of H_V1?

The clearest function of proton channels in phagocytes (neutrophils, macrophages, and eosinophils) is to facilitate and sustain NADPH oxidase (or "Nox2") activity. Detection of a foreign invader (e.g., a bacterium) by the phagocyte triggers a complex series of biochemical and cellular process culminating in phagocytosis of the detected material. A rapid biochemical consequence of the detection mechanism is the assembly, from its several components, of Nox2 in the phagosome membrane. Nox2 uses intracellular NADPH as a source of electrons, which it translocates across the phagosome membrane to produce reactive oxygen species that are released into the forming or mature phagosome. Electron translocation by Nox2 depolarizes the membrane potential,[65] while NADPH catabolism, which releases $NADP^+$ and H^+,[8,66] decreases the intracellular pH.[65,67–70] Proton efflux through H_V1 compensates for both the membrane depolarization and the pH change.[10,11,64,65,68,69,71–73]

Another consequence of the detection of foreign entities by phagocytes is a radical change in the gating kinetics of H_V1, which turns out to be a significant factor contributing to the activation of H^+ efflux. Four properties of hH_V1 change: opening occurs more quickly, closing slows, the maximum conductance $g_{H,max}$ increases, and the g_H–V relationship shifts negatively by 40 mV.[71,74–77] This transformation of H_V1 properties is termed the "enhanced gating mode." As a result of the four changes in H_V1 behavior, each of which promotes H^+ efflux, during enhanced gating the channel opens sooner and thereby improves the efficiency of NADPH oxidase activity by 15–20%.[78] Is it possible that enhanced gating reflects conversion

of the normally dimeric hH_V1 to monomeric status? This suggestion[3] has not yet been resolved beyond doubt, but the bulk of evidence suggests that this is not the case.[79]

Monomeric H_V1 and H_V1 in enhanced gating mode share one property: monomeric H_V1 open several times faster than dimeric channels.[3,5,24,52] Similarly, during enhanced gating, H_V1 opens four to six times faster than in resting cells.[12,71,74–77,80,81] Three other properties evident in the enhanced gating mode differ from those of monomeric constructs. (1) The position of the g_H–V relationship appears to be 10–15 mV more positive in monomeric constructs than WT dimeric channels.[52,53] In enhanced gating mode, the g_H–V relationship shifts in the opposite direction, negatively by ~40 mV.[71,74–77,80,81] (2) During enhanced gating, the Zn^{2+} sensitivity of hH_V1 is not changed,[52] whereas the Zn^{2+} sensitivity of the C-truncated construct was weaker than that of the dimer.[24] (3) During enhanced gating, activation of hH_V1 continues to have a sigmoid time course, in contrast to the exponential activation of the monomer.[24] On balance, the differences in channel properties during enhanced gating mode compared to monomeric constructs appear to outweigh the similarities. In another supporting piece of evidence, Petheő *et al.*[26] did not observe any difference in DSS cross-linking potential when they pretreated human neutrophils with PMA, which effectively induces the enhanced gating mode.

An alternative explanation is that phosphorylation of hH_V1,[82] specifically at Thr^{2983} is responsible for converting the resting channel into enhanced gating mode. The PKC activator PMA produces enhanced gating[12,15,16,74–77,80,82–84] that is prevented or reversed by PKC inhibitors such as staurosporine or GF109203X (GFX).[12,15,76,77,82–84] Other activators of enhanced gating including arachidonic acid and lipopolysaccharide act at least partially through PKC.[81,82,84]

6.2. Might the differences in properties of monomeric and dimeric constructs provide clues to the functional importance of dimerization?

The main differences between WT dimeric H_V1 and monomeric constructs include four properties: (a) activation (channel opening) is approximately five times slower in WT dimeric channels, (b) WT channel opening occurs with a sigmoid rather than exponential time course, (c) the WT g_H–V relationship is 10–15 mV more negative, and (d) the WT voltage dependence is twice steeper. We may for now discount the difference in opening time course (b) and the relatively subtle change in the position of the g_H–V

relationship (c). Although there are certain situations in which these changes could have functional ramifications—in excitable cells like cardiac muscle, for example, a 10 mV shift of a g–V curve has profound physiological significance[85]—in the absence of detailed knowledge about the precise situations in which H_V1 operate in most of the many cells where they exist, it seems pedantic to focus on small differences. The faster opening (a) and steeper voltage dependence (d) do, however, seem important enough to warrant speculation.

As discussed earlier, phagocytes, especially neutrophils and eosinophils, use proton channels during the respiratory burst, when Nox2 is active. The best understood role of H_V1 in this situation is to compensate charge,[65] to prevent excessive depolarization that would otherwise result from the activity of the electrogenic Nox2. Nox2 activity tends to occur with a delay of several seconds or even minutes, depending upon the stimulus, and may continue for hours.[86] Thus, it may be that the ability of H_V1 to open rapidly may not have much value to these cells. The phagocyte "cares" only that enough H_V1 open as soon as Nox2 is active to limit the depolarization. From the vantage point of the phagocyte, there is no rush, because Nox2 turns on slowly and remains active for a long while. In fact, in these nonexcitable cells, proton channel opening is quite slow, compared with the kinetics of gating of most ion channels, and compared with the opening of H_V in other species, such as snail neurons.[63,87,88]

On the other hand, H_V1 opening plays a major role in limiting the extent of the depolarization that occurs when Nox2 is active; membrane depolarization, especially beyond +50 mV, directly inhibits Nox2 activity.[10] To "help" Nox2, H_V1 should open within a relatively negative voltage range. The enhanced gating mode, which shifts Hv1's g_H–V relationship negatively by 40 mV, clearly facilitates this goal. Enhanced gating improves the efficiency of Nox2 by 15–20%, because to open enough channels to compensate Nox2, requires 24–30 mV less depolarization in human neutrophils and eosinophils than would be needed if there were no enhanced gating mode.[78] We have already seen that enhanced gating mode does not seem to represent a change in the dimerization state of H_V1.

The steep voltage dependence of H_V1 also facilitates Nox2 activity. Cooperative gating increases the steepness of the voltage dependence of ion channels.[89] The cooperative gating of the hH_V1 dimer results in a doubling of the voltage dependence compared to the monomer.[22] The result is that substantially less depolarization is required to open a given number of proton channels. One might argue that the cell could enable sufficient

activation of g_H simply by shifting the g_H–V relationship negatively, but once enhanced gating is in effect, $V_{threshold}$ is already perilously close to E_H. The cell "wants" H_V1 to turn on rapidly once there is an outward electrochemical gradient for H^+, but it definitely does not want H_V1 to open when the gradient is inward. The main function of H_V1 in most cells is acid extrusion—cells continually produce metabolic acid that they must eliminate; this is the "central problem of pH_i regulation."[90] For this reason, it would be self-destructive to have a constitutively active g_H, because this would act as a proton leak that would flood the cell with unwanted H^+. The most optimal possible design is to poise $V_{threshold}$ near E_H and then to have strong dependence of P_{open} upon depolarization above this point. Nature has produced the most efficient possible mechanism.

It would be interesting to know why H_V in some species appear to be monomeric. However, because we know the functions and the situations in which the channels are active only in the most general terms, it seems premature to attempt to predict why monomeric behavior would be preferable.

7. CONCLUSIONS

In most of the species where it has been identified, H_V1 exists as a dimer. Dimerization is driven and enforced mainly through coiled-coil interactions at the C-terminus; H_V1 sequences from a few single-celled organisms do not have a predicted coiled-coil region and so may exist as monomers. Dimerization in H_V1 provides the opportunity for a cooperative gating mechanism that gives rise to a steeply voltage-dependent conductance. This property appears to be important in the most intensively studied function of H_V1, which exists in human phagocytes, where steep voltage dependence provides optimum compensation for Nox activity and phagocyte function. A number of questions regarding H_V1 dimerization remain open, including the nature of the dimer interface, the mechanism of cooperativity, the impact of multimeric state on trans-acting factors (e.g., kinases) of H_V1, possible effects of transitions between monomeric and multimeric states, and implications of cooperative gating in cell types other than phagocytes.

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