Biochimica et Biophysica Acta 1838 (2014) 382–387

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Mapping of sites facing aqueous environment of voltage-gated proton channel at resting state: A study with PEGylation protection

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ARTICLE INFO

Article history:
Received 26 July 2013
Received in revised form 30 September 2013
Accepted 1 October 2013
Available online 16 October 2013

Keywords:
Ion channel
Proton
Voltage
Membrane potential
Gating
pH

ABSTRACT

Hv1 (also named, voltage-sensor only protein, VSOP) lacks an authentic pore domain, and its voltage sensor domain plays both roles in voltage sensing and proton permeation. The activities of a proton channel are intrinsic to protomers of Hv1, while Hv1 is dimeric in biological membranes; cooperative gating is exerted by interaction between two protomers. As the signature pattern conserved among voltage-gated channels and voltage-sensing phosphatase, Hv1 has multiple arginines intervened by two hydrophobic residues on the fourth transmembrane segment, S4. S4 moves upward relative to other helices upon depolarization, causing conformational change possibly leading to the formation of a proton-selective conduction pathway. However, detailed mechanisms of proton-selectivity and gating of Hv1 are unknown. Here we took an approach of PEGylation protection assay to define residues facing the aqueous environment of mouse Hv1 (mHv1). Accessibilities of two maleimide molecules, N-ethylmaleimide (NEM) and 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS), were examined on cysteine introduced into individual sites. Only the first arginine on S4 (R1: R201) was inaccessible by NEM and AMS in mHv1. This is consistent with previous results of electrophysiology on the resting state channel, suggesting that the accessibility profile represents the resting state of mHv1. D108, critical for proton selectivity, was accessible by AMS and NEM, suggesting that D108 faces the vestibule. F146, a site critical for blocking by a guanidinium-reagent, was accessible by NEM, suggesting that F146 also faces the inner vestibule. These findings suggest an inner vestibule lined by several residues on S2 including F146, D108 on S1, and the C-terminal half of S4.

1. Introduction

Hv1 (also named, "voltage-sensor only protein: VSOP") [1,2] is the voltage-gated hydrogen (Hv) channel. Hv1 is expressed in blood cells, sperm, microglia, and airway epithelium, playing a critical role in the homeostasis of intracellular pH and membrane potential [3,4]. It is involved in diverse biological events, including host defense and sperm motility. Hv1 has also been claimed to be involved in pathological events such as neuronal cell death in brain ischemia [5] and exacerbation of cancer [6]. It is also widely conserved among species and diverse biological functions such as calcification of marine algae [7] and light emission in marine phytoplankton [8–10] have been suggested. In Hv1, four transmembrane segments, with significant homology to the voltage sensor domain of voltage-gated ion channels, confer both ion selective permeation and voltage-dependent gating. Hv1 forms dimers both in heterologous expression systems and in native cells [11–14]. However, most of the basic properties are intrinsic to a protomer: the protein lacking both N-terminal and C-terminal regions fails to assemble as a dimer but exhibits activities as Hv channels [12,13].

Since Hv1 lacks an authentic pore domain, it is a fundamental question where proton permeates through. It is also a challenging question how the motion of voltage sensor regulates proton permeation in Hv1. In particular, gating of Hv1 is not only voltage-dependent but also pH dependent [15]. Activation voltage range is shifted dependent on the pH difference across the cell membrane (ΔpH). How Hv1 senses ΔpH for both environments across the cell membrane to regulate gating remains a mystery.

The proton permeation pathway has so far been studied both by electrophysiology in heterologous cell expression [16–20] and molecular dynamics simulation [18,21,22] using a homology model based on X-ray crystal structures of voltage-gated potassium channels and sodium channels all of which are predicted to be in activated state. Two molecular dynamics simulation studies based on the homology model with the voltage sensor of Kv channel proposed a model where static water molecules exist longitudinally over 5 to 10 Å in the central crevice forming a water wire for proton conduction [18,21]. Ramsey et al. made a series of point mutations on amino acids with titratable

Abbreviations: NEM, N-ethylmaleimide; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid

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http://dx.doi.org/10.1016/j.bbamem.2013.10.001

residues and failed to find any amino acid which is indispensable for proton conduction [18]. Combined with findings of molecular dynamics simulation, it was claimed that stationary water molecules in the constricted region confer proton selectivity and pH sensitivity of channel gating [18].

Recent electrophysiological studies have highlighted several amino acid residues for proton selective permeation. Aspartic acid on S1 in Hv1, D112 in human Hv1 (hHv1), corresponding to D108 in mouse Hv1 (mHv1), D160 in ascidian Hv1 (Ci-Hv1) or D51 in dinoflagellate Hv1 (kHv1) [10], is critical for proton selectivity, since mutation of D112 in hHv1 or D51 in kHv1 makes Hv1 permeable to anions [19,10]. R3 residue (R211 in hHv1 or R207 in mHv1) has also been proposed to be critical for proton-selective permeation: mutation of R3 residue makes hHv1 leaky to guanidinium ions of which geometry resembles that of the arginine residue [20]. This study proposed that D112 (D108 in mHv1) forms a salt bridge with arginine on R3 in the activated state. However, this idea contradicts with a finding that a truncation downstream of R2 lacking the R3 of mHv1 did not eliminate proton-selective permeation [16]. A recent study of zinc accessibility to histidine replacing arginine on the R3 position of hHv1 in an activated state with molecular dynamics simulation based on a homology model were also inconsistent with this idea and rather suggests that the salt bridge between D112 (D108 in mHv1) and R2 (R208 in hHv1 or R204 in mHv1) is important for proton conduction [22]. A discovery of guanidinium-related compound, 2-guanidinobenzimidazole (2GBI, m.w. 184), as an open channel blocker for Hv1 indicates the presence of vestibule that is continuous to the cytoplasm. Mutation of F150 in hHv1 makes a shift of dose–response curve of blocking by 2GBI, suggesting that this residue is critical for binding to 2GBI. In sequence alignments, F150 of hHv1 corresponds to phenylalanine in the gating charge transfer center [23] conserved among all voltage-gated ion channels and voltage-sensing phosphatases, suggesting that 2GBI enters the boundary between the aqueous environment and the hydrophobic core in Hv1.

Currently, no atomic structure of Hv1 has been resolved. We have previously applied a method of biochemical cysteine scanning, using two types of maleimide reagents, mal-PEG and AMS [16]. This "PEGylation protection assay" [24] with heterologous cell expression with mHv1 in HEK293 cells [16] led to a finding that the inner half of S4, downstream of R2, faces an aqueous environment. Such profile matches with the results of a cysteine accessibility study with an MTS reagent on the sea squirt ortholog of Hv1, Ci-Hv1, under resting condition [25], suggesting that analysis by PEGylation protection assay provides information of the configuration of Hv1 in the resting state. In this study, the same method [16] was extended to all residues in transmembrane helices of mHv1. Besides AMS, NEM, a smaller maleimide molecule, was also utilized for accessibility and compared with the results with AMS. This comparison provides insight into the size of the aqueous environment of transmembrane helices. The reactivity/accessibility of cysteine residues could also be restricted by close contact between helices within or between protomers and by the low dielectric nature of the local environment. We found that D108 (D112 in hHv1), a critical residue for proton selectivity, can be accessed both by NEM and AMS, supporting a model that D108 faces a relatively large vestibule to which both anions and cations are accessible. In addition, R201, D181, and E115 (R205, D185, E119 in hHv1) were both inaccessible by NEM and AMS, raising a possibility that R201 forms a salt bridge with D181 or E115 forming a barrier together with the hydrophobic core region in resting state.

2. Materials and methods

2.1. Plasmids and heterologous expression of mHv1

The point mutants were made using a QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instruction. Wild-type or mutant mHv1 cDNAs are subcloned into the PstI–SacII site of pIRES2-EGFP (Clontech) and transfected into HEK293 cells with polyFect (Qiagen). The residue number denotes the position in mHv1. The number incremented by four corresponds to the residue number in hHv1 (for example, R201 in mHv1 is R205 in hHv1).

2.2. PEGylation-protection assays

PEGylation protection assay in heterologous cell expression was performed as previously described [16]. In this assay, both AMS and NEM can permeate through cell membranes, and a site exposing to an aqueous environment both at the extracellular side and at the intracellular side can be detected by accessibility either by AMS or NEM. Basically, experiments followed the original method by Deutsch's group [26,24]. Briefly, HEK293 cells were washed with PBS, treated with 10 mM AMS or 5 mM NEM for 30 min at room temperature, and homogenized. The cell homogenates were pelleted, washed twice in PBS, and solubilized in the buffer [2% (wt/vol) SDS, 6 M urea, and 15 mM Tris, pH 7.4]. Aliquots of the resultant solution were then incubated with 5 mM mal-PEG (Sigma-Aldrich) for 30 min at room temperature, after which the proteins were treated with SDS/PAGE loading buffer [2% (wt/vol) SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue and 0.0625 M Tris–HCl, pH 6.8] and separated on 12.5% SDS/PAGE. Any reducing reagent was not included in the samples in all steps before this step. After electrophoresis, the separated proteins in the gel were electrophoretically transferred to Immobilon-P membranes (Millipore). A polyclonal rabbit anti-mHv1 antibody generated against the amino acids Met-1-Ala-72 in the N terminus of mHv1 was used for detection [16]. Horseradish peroxidase-conjugated anti-rabbit IgG from donkey (Amersham Pharmacia) was used as the secondary antibody.

At least three experiments were performed on individual sites. Quantification of western blot signal was performed by Light Capture II analyzer (ATTO Co.).

Among 108 mutants, the functionality of 12 mutant proteins was verified by whole cell patch clamp recording in HEK293T cells (L197C to I208C).

3. Results

Following the previously established method [16], AMS/NEM accessibility was examined on HEK293 cells transfected with mHv1 with an individual site replaced by cysteine. In this method, cells expressing the cysteine mutant were exposed with AMS or NEM, a small maleimide reagent, followed by the addition of mal-PEG, a large maleimide molecule. The addition of mal-PEG can be detected by the band shift due to the increase of molecular weight by western blot. When AMS or NEM can be accessed to the cysteine site before mal-PEG treatment, binding of mal-PEG can be protected leading to the absence or decrease of the band shift in western blot. All mutations were made in the cysteine-less background: native cysteine residues in S1 (C103) and coiled-coil region (C245) were mutated to serine. A total of 108 sites, including all amino acids in transmembrane helices and flanking sites in the cytosolic or extracellular loops, were mutated individually to cysteine and accessibility by AMS or NEM was tested.

3.1. Accessibility by AMS

Hv1 is known to form a dimer [11–13]. We did not observe a dimeric band in the Western blot as in the previous study probably because a reducing reagent was contained in the samples at the step of SDS/PAGE [16]. Results show that the C-terminal region of S4, distal from A206, was accessible by AMS, consistent with the previous report [16]. Some residues in S3a are also accessible by AMS. On the other hand, S1 and S2 are mostly inaccessible by AMS.

An exception was D108 on S1, which is known to be critical for proton-selective permeation [19] (Figs. 1A, 3A).

3.2. Accessibility by NEM

Results showed that a larger number of sites were protected from PEGylation by pretreatment with NEM than with AMS, as expected from its smaller molecular weight than that of AMS (Fig. 1A, B). Many residues on S4, which were AMS-inaccessible, were NEM-accessible. The region L199 to W203 and V205 of S4 were NEM-inaccessible. Eight amino acids on S3, which were inaccessible by AMS, were accessible by NEM, whereas residues in the core region from V175 to L182 were inaccessible by NEM. In S1, I102, D108, L118, and D119 are accessible by NEM, and in S2, F146, E149, K153 and F155 are accessible by NEM. As in results with AMS, V105 or L104, one helix-turn downward from D108, and L111 or V112, one helix-turn upward from D108, were inaccessible by NEM.

3.3. Mapping of AMS/NEM accessibility

The extent of accessibility by NEM was quantified by the ratio between a mal-PEG positive band (upper band) and a negative band (lower band) and indicated by different colors (blue 0–5%, green 5–20%, orange 20–60%, red 60–100%) (Fig. 2). The result was visualized onto the membrane topology by SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/) as all-or-none fashion (blue: accessible, red: inaccessible) (Fig. 3). The decision for “accessible site” or “inaccessible site” was made as in the following; the inaccessible site (red) was taken by the detection of a mal-PEG bound signal (upper band) and the accessible site (blue) was taken by the absence of a mal-PEG bound signal. Quantification was done from three blots.

Results show that the C-terminal region of S4 is accessible by NEM over a long stretch, and only R201 among three arginines in S4 is inaccessible by NEM. Only 6 residues of the S4 helix are shielded in the membrane. D108 is both accessible by NEM and AMS. Some residues, such as S139, S177, D181 (partially accessible), E115, and R201, were not accessible either by NEM or AMS, despite the hydrophilic nature of residues.

F146, a site postulated to be located at the boundary between the constrictive proton permeating pathway and the inner vestibule [21], was NEM-accessible, but AMS-inaccessible. V112, I142, and L143 which have been postulated to form a hydrophobic cluster located slightly upper (extracellular) than F146 in the molecular model [21] were inaccessible by NEM and AMS.

4. Discussion

Hv1 contains the voltage sensor domain lacking an authentic pore domain and its monomer has dual roles: proton-selective permeation and gating. In the present study, to gain insights into the molecular structure of Hv1, a PEGylation protection assay was applied. Using two maleimide reagents with a distinct molecular size enabled us to predict a profile of aqueous environment of mHv1. Experiments with AMS on S4 reproduced our previous results of accessibility to amino acids on S4 [16]. NEM accessibility showed a more detailed profile of S4: R1 was shielded both from NEM and AMS, whereas R2 was accessible by

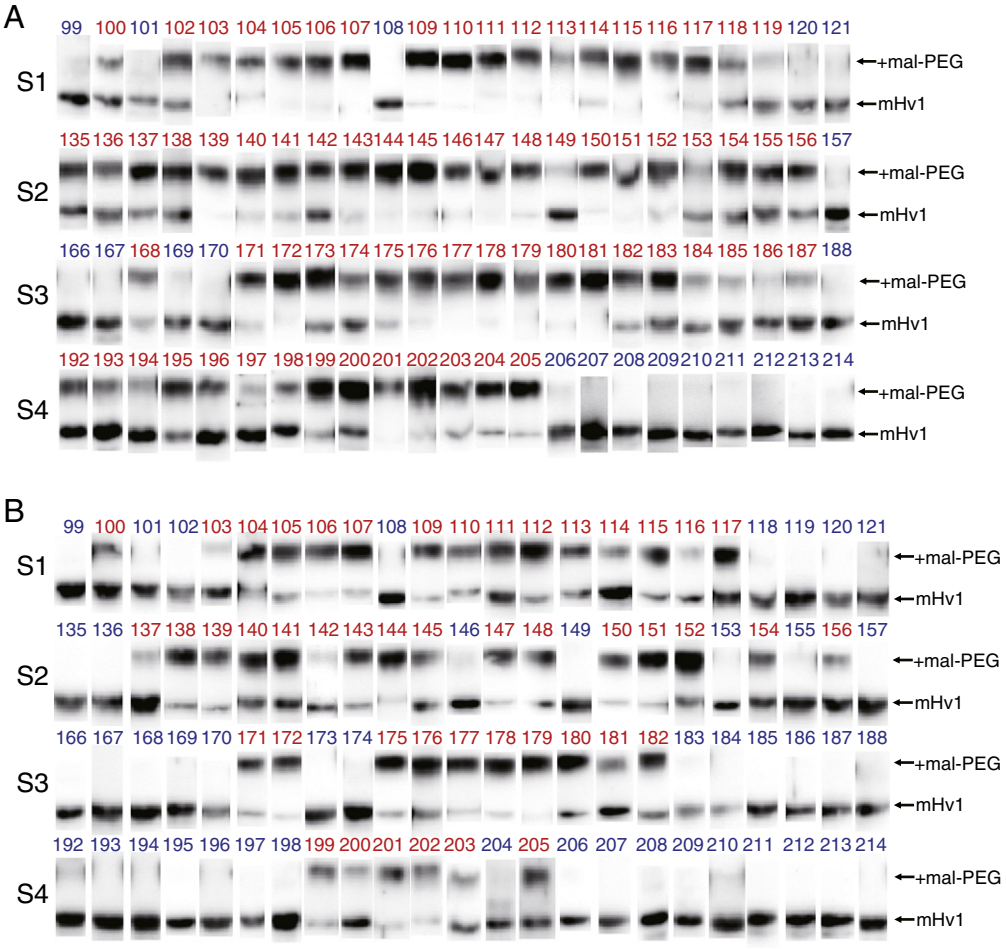

Fig. 1. Blots of PEGylation protection assay with AMS (A) and NEM (B) on individual residues of transmembrane helix, S1 to S4, of mHv1. Residue number of site where PEGylation with mal-PEG was protected by AMS (A) or by NEM (B) is labeled by a blue letter. Unprotected site (namely AMS- or NEM-inaccessible site) is labeled by a red letter.

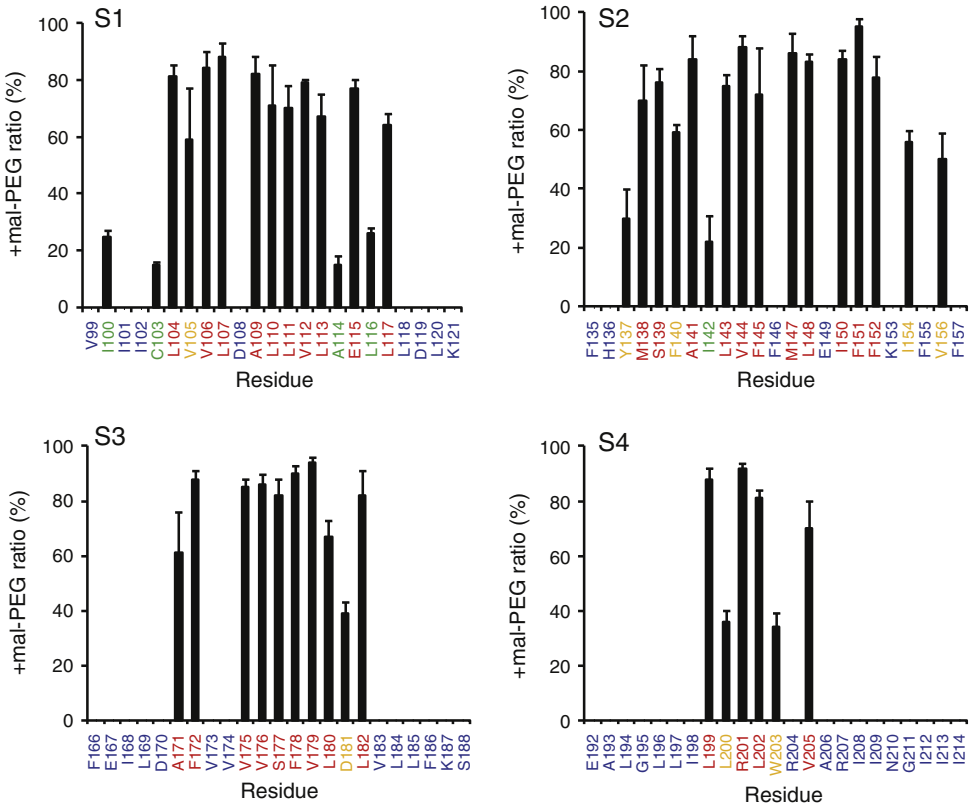

Fig. 2. Quantification of the extent of access of mal-PEG (unprotected proportion) pretreated with NEM as the protector of mal-PEG binding. The extent of accessibility by NEM was quantified by the ratio between the mal-PEG positive band (upper band) and the negative band (lower band) and indicated by different colors (blue 0–5%, green 5–20%, orange 20–60%, red 60–100%).

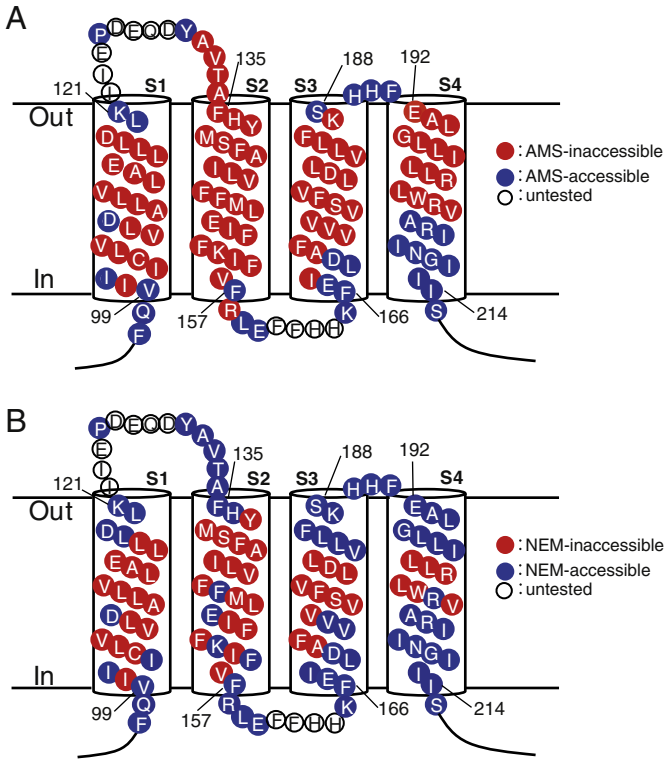

Fig. 3. Maps of NEM/AMS accessibility for mHv1. (A) Map of AMS accessibility. (B) Map of NEM accessibility.

NEM but not by AMS, and R3 was accessible both by NEM and AMS. This indicates that R3 is exposed to a wide aqueous environment, whereas R2 is in a narrow aqueous environment, and R1 is embedded in a shielded core region. In the electrophysiological study of accessibilities of an MTS reagent to Ci-Hv1 in heterologous expression [27,25], R2 was accessible from the extracellular side in an activated state and R3 was inaccessible from the intracellular side in the activated state. In resting state, R2 and R3 are accessible from the intracellular side [27] (Table 1). These profiles by cysteine accessibility with an MTA reagent in resting state match with the results with NEM in the present study, indicating that our results reflect the profile of mHv1 at the resting state.

Based on the experiments with NEM, it is concluded that five residues of S4 helix are shielded in the membrane from the aqueous environment. This stretch of five residues corresponds to 7.5 Å if it takes an α-helix, or 10 Å in the case of a 3_{10} helix. In other helices, eight residues (S3) or nine residues (S1 and S2) were shielded from the aqueous environment.

It should be noted that some hydrophilic residues were not accessible by NEM. These include S139, S177, D181 (partially accessible), E115 and R201. This raises a possibility that R201 forms a salt bridge with D181 and/or E115. S139 and S177 probably provide a hydrogen bond which may also be preserved in the activated channel to support the proton conducting pathway.

D108 is critical for proton selective permeation, and mutation of this site renders channel anion-permeable [19], raising a possibility that cations are excluded at the ion permeating pathway up to D108 and do not get access to the vicinity of D108 [4]. However, AMS accessibility to D108 suggests that cations should be able to get access to D108 in the resting state. Therefore it is more likely that the local structure involving D108, rather than the pathway up to D108, determines the exclusion of other cations than proton.

Table 1
Comparison of accessibility with previous studies.

Residue number of hHv1	Residue number of Ci-Hv1	Accessibility by MTS reagent				NEM-accessibility	Residue number of mHv1
		Upstate (activated state)		Downstate (resting state)			
		Ext	Int	Ext	Int		
E196	A246	+[a]		−[a]		+	E192
L198	I248	+[a]		−		+	L194
I202	V252	+[a]		−[a]		+	I198
L204	L254					−	L200
R205	R255	+[b]				−	R201
L206	L256	−[a]		−	−[a]	−	L202
R208	R258	+[c,b]	−[c]	−[c]	+[c]	+	R204
V209	V259	−[a]			−[a]	−	V205
R211	R261	−[a]	+[b]			+	R207
I212	I262		−[a,c]		+[a,c]	+	I208
N214	N264		−[a]		+[a]	+	N210

+ means accessible site, − means inaccessible site.
Ext: external accessibility.
Int: internal accessibility.
[a] [25] Cysteine scanning with accessibility to MTS reagent done by electrophysiology on Ci-Hv1.
[b] [22] Histidine scanning of zinc sensitivity done by electrophysiology on hHv1.
[c] [27] Cysteine scanning with accessibility to MTS reagent done by electrophysiology on Ci-Hv1.

F146 has recently been shown to be critical for binding to the guanidinium-related blocker, 2GBI [28]. A mutation of F146 makes the channel more sensitive to 2GBI, suggesting that 2GBI binds to F146 or its vicinity. 2GBI blocks a channel only when a channel becomes activated, reminiscent of the effects of quaternary ammonium ions in potassium channels [29]. In this study, F146 was accessible by NEM. In S2, E149, K153 and F155 are also NEM-accessible, but AMS-inaccessible, suggesting that these residues also face an aqueous crevice up to F146. Upon activation of Hv1, the local structure involving F146 is changed in a state-dependent manner to bind 2GBI. The open channel blocking action of 2GBI on Hv1 may suggest the presence of some gate in intracellular vestibule as established in K^+ channel [29]. However, estimated from the molecular size of 2GBI compared with NEM, 2GBI should be docked in the vicinity of F146 in the resting state. This suggests that no gate is present in the cytoplasmic side distal to F146 and the architecture for proton selectivity itself serves as the gate. Interestingly, V105 or L104 one helix-turn downward from D108 on S1 is expected to face the same side as D108 within the protomer, but it was inaccessible by NEM. This region may have a low dielectric environment or have a narrow space by interacting with adjacent residues of other helices. It should be cautioned that these arguments depend on the assumption that cysteine mutation introduced into an individual site does not drastically alter the position of the residue as compared with the position in a native channel. A detailed three-dimensional spatial profile must await the success of direct structural analysis at high resolution.

In summary, we obtained a gross view of the aqueous environment of mHv1 most likely in the resting state. Results support the view that Hv1 has a wide inner aqueous vestibule which is lined by four helices. Five sequential amino acids on S4, including R1 (R201 in mHv1, R205 in hHv1), are shielded from both extracellular and intracellular aqueous space. In this NEM-inaccessible core, R1 arginine on S4 probably forms a salt bridge with the carboxyl group of D181 (D185 in hHv1) or E115 (E119 in hHv1) in closed mHv1. R2 arginine and downstream sites on S4 face an inner aqueous vestibule. F146 (F150 in hHv1) and D108 (D112 in hHv1) are located facing or in the vicinity of the inner aqueous vestibule. These findings will provide insights into the structural basis for proton permeation and gating of Hv1.

Author contributions

T.K. and Y.O. conceived and designed the research and wrote the paper. T.K. performed all experiments. All authors approved the paper.

Acknowledgements

We thank all members of the laboratory for helpful discussion. This work was supported by the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) [grant numbers 21229003 and 21770171 (to Y.O. and T.K.)].

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2013.10.001.

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