Arrangement and mobility of the voltage sensor domain in prokaryotic voltage-gated sodium channels

Takushi Shimomura¹, Katsumasa Irie¹,², Hitoshi Nagura¹, Tomoya Imai³ and Yoshinori Fujiyoshi¹,²

Running head: Domain arrangement of prokaryotic NaVs

¹Department of Biophysics, Graduate School of Science, Kyoto University, Oiwake, Kitashirakawa, Sakyo-ku, Kyoto 606-8502; ²Japan Biological Informatics Consortium, Oiwake, Kitashirakawa, Sakyo-ku, Kyoto 606-8502; and ³Research Institute for Sustainable Humanosphere (RISH), Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan

Address correspondence to Yoshinori Fujiyoshi, Department of Biophysics, Graduate School of Science, Kyoto University, Oiwake, Kitashirakawa, Sakyo-ku, Kyoto, Japan, 606-8502. Tel.:+81-75-753-4216; Fax: +81-75-753-4218; E-mail: yoshi@em.biophys.kyoto-u.ac.jp

Prokaryotic voltage-gated Na channels (NaVs) form homotetramers with each subunit contributing six transmembrane α-helices (S1-S6). Helices S5 and S6 form the ion conducting pore, and helices S1-S4 function as the voltage sensor with helix S4 thought to be the essential element for voltage-dependent activation. Although the crystal structures have provided insight into voltage-gated K channels (KVs), revealing a characteristic domain arrangement in which the voltage sensor domain of one subunit is close to the pore domain of an adjacent subunit in the tetramer, the structural and functional information on NaVs remains limited. Here, we show that the domain arrangement in NaChBac, a firstly cloned prokaryotic NaV, is similar to that in KVs. Cysteine substitutions of three residues in helix S4, Q107C, T110C, and R113C, effectively induced inter-subunit disulfide bond formation with a cysteine introduced in helix S5, M164C, of the adjacent subunit. In addition, substituting two acidic residues with lysine, E43K and D60K, shifted the activation of the channel to more positive membrane potentials and consistently shifted the preferentially formed disulfide bond from T110C/M164C to Q107C/M164C. Because Q107 is located closer to the extracellular side of helix S4 than T110, this finding suggests that the functional shift in the voltage dependence of activation is related to a restriction of the position of helix S4 in the lipid bilayer. The domain arrangement and vertical mobility of helix S4 in NaChBac indicate that the structure and the mechanism of voltage-dependent activation in prokaryotic NaVs are similar to those in canonical KVs.

Voltage-gated ion channels play essential roles in electric signaling, muscle contraction, and other important physiologic processes (1). Mammalian voltage-gated Na channels (NaVs) are formed by a single, long polypeptide (~2000 amino acids) that contains four homologous domains (2). Prokaryotic NaVs are simpler than mammalian NaVs, comprising shorter polypeptides of ~300 amino acids that form homotetramers (3-6). Each subunit, corresponding to one homologous domain in mammalian NaVs, contains six transmembrane α-helices (S1-S6). Helices S5 and S6 form the ion conducting pore in the center of the tetrameric channel, and helices S1-S4 form voltage sensors that surround the pore domain and detect the membrane potential. Helix S4 features a series of positively charged residues that are essential for voltage-dependent gating (7, 8). It is thought that changes in the membrane potential cause some of these charges to move vertically in the lipid bilayer (9).

NaChBac is a prokaryotic NaV cloned from Bacillus halodurans. Its function has been studied by expression in mammalian cells and confirmed to be a Na⁺ selective channel (3), providing insight into gating charge movements related to voltage-dependent gating (10), and C-type inactivation (6, 11). Different prokaryotic NaVs differ in their voltage dependence and ion conduction kinetics (5, 6). The structural simplicity and functional diversity of prokaryotic NaVs make them an ideal model for studying the structure and function of other NaVs.
The best-studied voltage-gated ion channels are voltage-gated K channels (KVs). The arrangement of subunits in KVs tetramers was initially investigated by introducing double cysteine mutations at the extracellular side of helices S4 and S5 in the Shaker channel from *Drosophila melanogaster* (12-14). Some double cysteine mutation pairs result in the formation of inter-subunit disulfide bonds, showing that helix S4 of one subunit is in close proximity to helix S5 of an adjacent subunit. The proximity of the residues identified in these studies was subsequently verified with the crystal structures of the rat Kv1.2 and Kv1.2/2.1 chimera channels (15, 16), which showed that helix S4 of the voltage sensor domain indeed faces helix S5 of the pore domain of an adjacent subunit in the Kv tetramer.

The domains in tetramers formed by prokaryotic NaVs are thought to have an arrangement similar to that of tetrameric KVs. Confirming that KVs and prokaryotic NaVs have similar domain arrangements would allow findings obtained from analyses of prokaryotic NaVs to be generalized to all tetrameric voltage-gated ion channels. While recent distance measurements by luminescence energy transfer suggested that NaChBac and KvAP, a prokaryotic Kv, share a similar subunit organization (17), direct evidence for a similar domain arrangement in KVs and prokaryotic NaVs is still missing.

Using the same double cysteine mutagenesis approach previously used for the Shaker Kv, we confirmed the proximity between helix S4 and helix S5 of an adjacent subunit in NaChBac. The double mutants that formed disulfide-bonded tetramers were consistent with previous results obtained for KVs. These results suggest that NaChBac has the same domain arrangement as KVs. We also show that substituting Q107, T110, and R113 in helix S4 with cysteine is the most efficient method of forming a disulfide bond with M164C in helix S5. Helix S4 is thought to move vertically during voltage-dependent activation (18-20). The finding that multiple residues in helix S4 can form disulfide bonds with the same residue in helix S5 indicates that helix S4 is very mobile in the vertical direction. To examine the relationship between the mobility of helix S4 and voltage-dependent activation, we assessed the effect of mutations in helices S1 and S2, which shifted the activation of NaChBac to a more positive membrane potential. The additional mutations resulted in M164C forming disulfide bonds preferentially with residues in helix S4 closer to the extracellular surface. These results demonstrate that the vertical position of helix S4 depends on the charges surrounding the voltage sensor domain and that changes in the electrostatic environment shift the voltage dependence of activation of NaVs.

**EXPERIMENTAL PROCEDURES**

*Molecular biology, protein expression and Western blot analysis—Construction of vectors carrying each NaChBac mutant, and expression of the wild-type and mutant NaChBac vectors were performed as previously reported with slight modification (6). For expression in *Escherichia coli*, cDNA encoding wild-type and mutant NaChBac was inserted into the pQE-80L vector (Qiagen) using the BamHI and HindIII sites, or into the pET-21b vector (Novagen) using the NdeI and SalI sites.*

NaChBac proteins were expressed using the pQE-80L vector in *E. coli* BL21 (Invitrogen). Cell cultures (15 mL) were grown for 72 h at 37°C after induction with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (Wako) to allow for disulfide bond formation. Cells were pelleted and resuspended in 1 ml TBS buffer (20 mM Tris-HCl [pH 7.4], 300 mM NaCl). The cell samples were mixed with electrophoresis sample buffer containing 20 mM iodoacetamide for non-reducing conditions or 2% β-mercaptoethanol for reducing conditions. After boiling for 5 min at 70°C, samples were analyzed by SDS-PAGE using 10% to 20% gradient gels (Wako). For Western blotting, proteins were transferred onto nitrocellulose membranes that were treated with blocking buffer (PBS buffer containing 0.25% gelatin, 2.5% BSA, and 0.01% NaN₃) for 30 min at room temperature and then incubated with Penta-His antibody (Qiagen) for approximately 12
h at 4°C. The antibody was detected by alkaline phosphatase-conjugated anti-mouse antibody (Promega) and developed with BCIP/NBT solution (Promega).

Quantification of the efficiency of disulfide bond formation—Mutant NaChBac proteins were expressed using the pET-21b vector in E. coli BL21. Collected cells were incubated for 1 h with 10 µg/ml egg lysozyme (Wako) in 1 mL TBS buffer, and then with 15 µg/ml DNaseI (Wako) and 15 mM MgSO₄ for 30 min at 4°C. Cells were sonicated, and insoluble material was removed by centrifugation at 12,000×g for 20 min at 4°C. The supernatant was subjected to SDS-PAGE, and the separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were treated with Blocking One solution (Nacalai Tesque) at room temperature for 30 min and incubated with a monoclonal antibody against NaChBac at 4°C for approximately 12 h. The antibody was detected by horseradish peroxidase-conjugated secondary antibody (Promega) and visualized using ECL Plus (GE Healthcare). The bands were scanned and quantified using an LAS-3000 image analyzer (FUJI FILM). Disulfide bond formation efficiency was calculated as the intensity of the band representing the disulfide-bonded tetramer divided by sum of the intensity of all bands. The efficiency of disulfide bond formation of each mutant was compared using Tukey’s test.

Electrophysiology—CHO-K1 or HEK293 cells were grown in DMEM (Invitrogen) supplemented with 10% FBS (BIOWHITTAKER), 100 units/ml penicillin (GIBCO), and 100 µg/ml streptomycin (GIBCO) at 37°C under a 5% CO₂ atmosphere. Cells were transfected with NaChBac and pEGFP DNA using the Calcium Phosphate Transfection Kit (Invitrogen) and plated on cover slips. HEK-293 cells were used to confirm the sodium currents of mutant channels under reducing and non-reducing conditions, and CHO-K1 cells were used to analyze the voltage dependence of activation. Cells were voltage-clamped with an EPC10 amplifier (HEKA Elektronik, Lambrecht, Germany). Whole-cell currents were recorded 24 to 48 h after transfection. The pipette solution was 10 mM HEPES-NaOH (pH 7.4), 105 mM CsF, 35 mM NaCl, and 10 mM EGTA; and the bath solution was 10 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 2 mM KCl, and 10 mM glucose. Patch pipette resistances were 2–3.5 MΩ. For measurements under reducing conditions, cells were treated with bath solution containing 5 mM DTT for 30 min. Data were collected using Pulse (HEKA). All experiments were conducted at 25 ± 2°C.

Current traces were recorded for a 500-ms depolarization from a holding potential of -120 mV. Voltage was stepped in 10 mV increments at 15-s intervals. The voltage dependence of NaChBac and mutant channel activation was determined from deactivation tail currents. To measure tail currents, a series of depolarizing test-pulse potentials from -120 mV holding potential were given in 10-mV steps for 40-ms followed by repolarization to -90 mV. The amplitudes of the tail currents were normalized to the maximal amplitude and fitted with a simple Boltzmann distribution, 1/ (1 + exp (V - V₁/₂)/κ) where κ is the slope factor and V₁/₂ is the voltage at which half-activation was reached.

RESULTS

Identification of paired cysteine mutations in helices S4 and S5 that lead to the formation of inter-subunit disulfide bonds—To investigate whether the domain arrangements in prokaryotic NaVs and KVs are similar, we systematically introduced double cysteine mutations in NaChBac to identify pairs that would allow the formation of inter-subunit disulfide bonds. A previous study of Shaker Kv showed that the cysteine substitutions of both R362 in helix S4 and A419 in helix S5 resulted in disulfide-bonded channel tetramers (12), indicating that residues R362 and A419 of the adjacent subunit are sufficiently close to each other to allow for the formation of a disulfide bond. If NaChBac has a similar domain arrangement as the Shaker channel, double cysteine substitution of the corresponding residues in helices S4 and S5 should allow for the formation of a disulfide-bonded NaChBac
tetramer (Fig. 1).

To determine what double cysteine mutants would form disulfide-bonded tetramers, we expressed them in *E. coli*, as it is an easy and convenient expression system that makes it feasible to screen a large number of mutants. Of note, NaChBac contains no cysteine residues. We first introduced a cysteine substitution for residue R113 in helix S4 of NaChBac, the residue that corresponds to residue R362 in the Shaker channel. We then generated 13 mutants with an additional cysteine substitution at positions 160 to 172 in helix S5 (Fig. 1), and the *E. coli* cells expressing the various NaChBac mutants were analyzed by Western blotting. While most mutants showed a band consistent with the molecular weight of a monomer (29 kDa, labeled 1 in Fig. 2A), the R113C/M164C double mutant migrated as a band of higher molecular weight (labeled 4 in Fig. 2A). With a molecular weight of ~120 kDa, approximately four times the molecular weight of a NaChBac monomer, the band should represent a disulfide-bonded tetramer. Thus, among the 13 residues tested, M164 was the residue in helix S5 closest to R113.

After identifying M164 in helix S5, we combined the M164C mutation with 12 cysteine mutations at positions 106 to 117 in helix S4 (Fig. 1). Western blot analysis of the double mutants showed that the T110C/M164C mutant was the most efficient in forming disulfide-bonded tetramers migrating at a molecular weight of ~120 kDa, while the R113C/M164C mutant was the second most efficient in forming disulfide-bonded tetramers (Fig. 2B). Of the 12 mutants tested, 8 showed a band corresponding to the disulfide-bonded tetramer (cysteine residues introduced from position 107 to 114). The Q107C/M164C mutant was slightly more efficient in forming disulfide-bonded tetramers than the other mutants, but less efficient than the T110C/M164C and R113C/M164C mutants. By combining the T110C substitution in helix S4 with the 13 cysteine substitutions in helix S5, we confirmed that M164C most efficiently formed a disulfide bond with T110C (Fig. S1). M164C also most efficiently formed disulfide bonds with V111C and L112C (Fig. S1). T110 and M164 thus appear to be the closest pair of residues between helices S4 and S5. SDS-PAGE analysis of wild-type NaChBac, the Q107C, T110C, R113C, and M164C single mutants, and the Q107C/M164C, T110C/M164C, and R113C/M164C double mutants showed that all variants migrated mainly as monomers under reducing conditions and only three double mutants migrated as tetramers under non-reducing conditions (Supplemental Fig. S2). These results confirmed that the Q107C/M164C, T110C/M164C, and R113C/M164C tetrats are stabilized by inter-subunit disulfide bonds between the substituted cysteines in helix S4 and M164C in helix S5.

Inter-subunit disulfide bonds formed in NaChBac T110C/M164C, and R113C/M164C mutants lock the channels in a non-conductive state—To assess the effect of inter-subunit disulfide bonds, we used whole-cell patch clamp analysis to compare the channel activity of the R113C/M164C double mutants with that of wild-type NaChBac. When transfected into mammalian cell lines, wild-type NaChBac generated a sodium current upon application of a series of test-pulses from a holding potential of -120 mV (Fig. 3A). Using the same regimen, R113C and M164C single mutants generated the same sodium currents under reducing and non-reducing conditions (Supplemental Fig. S3). In contrast, the R113C/M164C double mutant failed to generate a sodium current under non-reducing conditions, but treatment with 5 mM DTT restored the sodium currents of R113C/M164C (Fig. 3B). Although the mean amplitude of the R113C/M164C mutant with DTT-pretreatment was smaller, and the rate of current inactivation was slower than wild-type (Table 1), the sodium currents were generated normally, as in the wild-type. The same result was previously obtained for the T110C/M164C double mutant (21). These results indicate that positions 110 and 113 in helix S4 and position 164 in helix S5 are close enough to allow for the formation of inter-subunit disulfide bonds in the T110C/M164C and R113C/M164C double mutants. Thus, the inter-subunit disulfide bonds appear to lock the
The D60K and E43K mutations shift the activation of NaChBac to a more positive membrane potential—Cysteines introduced at multiple positions in helix S4 resulted in disulfide bond formation with M164C. This result may reflect the mobility of the voltage sensor domain. Because residues Q107, T110, and R113 would be positioned in a line along the axis of helix S4 with all side chains projecting from the helix in approximately the same direction but a turn of the helix apart, and the fact that double mutants Q107C/M164C, T110C/M164C, and R113C/M164C all formed disulfide-bonded tetramers indicates that helix S4 has substantial vertical mobility in the lipid bilayer.

To examine the relationship between the vertical mobility of helix S4 and the voltage-dependent activation of the channel, we investigated the effect of other mutations that affect the voltage dependence of NaChBac activation on the efficiency of disulfide bond formation in the three double mutants.

Residues E43 and D60 are located close to the extracellular surface in helices S1 and S2, respectively (Fig. 1B). The D60K mutation is known to shift the voltage dependence of NaChBac activation towards a more positive membrane potential (22, 23), and the crystal structure of the rat KV1.2/2.1 chimera channel shows that the residues corresponding to E43 and D60 interact with one of the positively charged residues in helix S4 (16). To determine the voltage dependence of the mutant channels, we used whole-cell patch clamp to measure deactivation tail currents. The mean amplitude of each mutant was generally smaller than that of the wild-type, but normally generated (Table 2 and Supplemental Fig. S4). A Boltzmann fit of the mean activation curve for the D60K mutant showed that the potential for 50% activation ($V_{1/2}$) was 42.0 ± 2.0 mV, a shift of +79 mV compared with that of wild-type NaChBac ($V_{1/2}$ of -36.6 ± 2.0 mV) (Fig. 4A). Similarly, $V_{1/2}$ for the E43K mutant was 18.0 ± 1.3 mV, a shift of +55 mV compared with the wild-type channel. The E43K mutation thus has a similar effect on the voltage dependence as the D60K mutation. The R113C mutation is known to cause a substantial shift in the voltage-dependent activation of NaChBac (24) and in the R113C/M164C double mutant this mutation is involved in the formation of a disulfide bridge. We therefore also analyzed the change in the voltage dependence of activation caused by the E43K or D60K mutations in the presence of an additional R113A mutation. $V_{1/2}$ for the R113A and D60K/R113A mutants were 2.59 ± 3.6 mV and 80.3 ± 0.9 mV, respectively, a difference between them of +78 mV (Fig. 4B). The $V_{1/2}$ for the E43K/R113A mutant was 24.5 ± 2.5 mV, a shift of +22 mV compared to the R113A mutant (Fig. 4).

Hence, the E43K or D60K mutations shift the voltage dependence of activation towards a more positive membrane potential, with or without the additional R113A mutation.

The D60K and E43K mutations change which residues in the double-cysteine mutants preferentially form disulfide bonds—We tested whether the D60K and E43K mutations would affect the disulfide bond formation of the three double-cysteine mutants, Q107C/R113A/M164C (QC), T110C/R113A/M164C (TC), and R113C/M164C (RC), in which residue R113 was neutralized by substitution to alanine or cysteine. The mutants were expressed in E. coli, and disulfide bond formation in the proteins was assessed by quantitative Western blot analysis (Fig. 5A). The efficiency of disulfide bond formation was determined as the intensity of the band representing the disulfide-bonded tetramer divided by the sum of the intensity of all bands (Fig. 5B). The QC mutant formed significantly fewer disulfide-bonded tetramers than the TC ($P < 0.01$) and RC ($P < 0.05$) mutants. Thus, neutralization of R113 by substitution to alanine or cysteine did not change the order concerning the efficiency with which the three double-cysteine mutants formed disulfide bonds. We next quantified the efficiency with which the three double-cysteine mutants formed disulfide bonds when we introduced an additional D60K or E43K mutation. The D60K/QC mutant formed disulfide bonds significantly more efficiently than the D60K/TC ($P < 0.01$) and D60K/RC ($P < 0.01$) mutants, and the E43K/QC mutant more efficiently than the E43K/RC mutant ($P < 0.05$). With both the E43K
and D60K mutations, the QC mutant was most efficient in forming disulfide bonds, followed by the TC mutant, and finally the RC mutant. These results suggest that in the presence of the E43K and D60K mutations, the residue in helix S4 closest to M164 in helix S5 is no longer T110 but rather Q107, and that the charges surrounding the voltage sensor domain affect the preferred vertical position of helix S4.

**DISCUSSION**

The domain arrangement in *NaChBac* is similar to that in *Kv*s—By generating double cysteine mutants, we confirmed that in *NaChBac*, helix S4 is close to helix S5 of an adjacent subunit. The observation of disulfide-bonded tetramers for the T110C/M164C and R113C/M164C mutants indicates that residues T110 and R113 in helix S4 are close to residue M164 in helix S5 of the adjacent subunit (Fig. 2), suggesting that the putative domain arrangement shown in Figure 1C is likely correct for prokaryotic NaVs. Our electrophysiologic analysis shows that the T110C/M164C and R113C/M164C mutants do not generate currents under non-reducing conditions, but activity is recovered under reducing conditions (Fig. 3) (21). The inter-subunit disulfide bonds presumably prevent conformational changes of the voltage sensor domains and keep the mutant channels in a non-conductive state. As alignment of the *NaChBac* and Shaker channel sequences shows that the T110C/M164C and R113C/M164C mutations in *NaChBac* correspond to the R362C/F416C mutations in the Shaker channel, the distances from the Cα carbon of F344 to those of R287, Q290, and R293 are 11.5, 8.4, and 8.1 Å, respectively. Thus, the location of residues Q107, T110, and R113 in *NaChBac* predicted from our disulfide cross-linking analysis is consistent with the position of the corresponding residues in the crystal structure of Kv1.2/2.1. These results further support a similar domain arrangement in *NaChBac* and Kv*s.*

The proximity of helices S4 and S5 of an adjacent subunit has also been observed in other six-transmembrane tetrameric ion channels. In KvAP, cysteine substitution of R117 and Y169, the residues corresponding to R113 and T163 in *NaChBac*, also results in the formation of inter-subunit disulfide bonds (25). Furthermore, the crystal structure of MlotiK1, a prokaryotic cyclic nucleotide-gated ion channel, showed that helix S4 is close to helix S5 of the adjacent subunit (26). The proximity of helix S4 to helix S5 of an adjacent subunit thus appears to be a characteristic feature of six-transmembrane tetrameric ion channels. The role of the interaction between helices S4 and S5 has not yet been firmly established, but some studies suggest that the interaction is required for channel function, particularly for voltage-dependent activation and inactivation (27-30).

**Helix S4 is highly mobile**—The efficient formation of disulfide bonds between M164C and cysteines introduced at positions 107, 110, and 113 indicates that all three of these residues on helix
S4 can come close to M164 in helix S5 (Fig. 2B). This result implies that helix S4 has substantial mobility, as much as 7.2 Å, the approximate distances of two helical turns, in the vertical direction. Of the 12 cysteine substitutions in helix S4 of NaChBac, 8 formed some disulfide-bonded tetramers with M164C (from Q107C to I114C) (Fig. 2B). Disulfide-bond formation between substituted cysteine residues is a well-established method for analyzing protein structure (31). Because of its helical structure, a rotational movement of helix S4 would allow residues in helix S4 other than Q107, T110, and R113 to form disulfide bonds with M164, but this appears to occur rarely. In contrast, of the 13 cysteine substitutions in helix S5 (from V160 to E172), only M164C fully formed disulfide bonds with residues in helix S4 (Fig. 2A and Fig. S1). This result indicates that helix S4 is highly mobile, appearing to move both vertically, and to some degree rotationally, in the lipid bilayer, whereas helix S5 was more static.

The vertical position of helix S4 is related to the voltage dependence of activation—Introducing the additional D60K mutation changed the preferentially formed disulfide bond in NaChBac from TC to QC (Fig. 5). Because the D60K mutation shifts the voltage dependence of activation towards a more positive membrane potential (Fig. 4), the change in the closest residues between helices S4 and S5 may be caused by the shift in activation potential. In the Shaker channel, the negative charge of the residue corresponding to D60 in NaChBac assists the charge transfer during voltage dependent activation by forming a salt bridge with a positively charged residue in helix S4 (32, 33). Substitution of D60 with lysine prevents the formation of this salt bridge, thus suppressing the vertical movement of helix S4. As a result, Q107, which is closer to the extracellular surface than T110, would become more accessible for disulfide bond formation with M164 and could be the cause of the shift in the voltage dependence of activation to a more positive membrane potential.

The effect of the E43K mutation on the voltage dependence of activation and the preferentially formed disulfide bridge was similar to those of the D60K mutation (Figs. 4 and 5). In the structure of the rat Kv1.2/2.1 chimera, the residue corresponding to E43 forms a salt bridge with the third positively charged residue in helix S4, whereas the residue corresponding to D60 interacts with the fourth positively charged residue (16). The role of residue E43 in voltage-dependent activation may thus be similar to that of D60, and the E43K mutation may thus also interfere with the vertical movement of helix S4. The role of negative charges in the vertical movement of helix S4 was recently demonstrated in NaChBac, as residues D60 and E70 were shown to interact sequentially with positive charges in S4 during voltage dependent activation (34). E43 also interacts with R113 and R116 during channel activation in NaChBac (35). The involved negative charges, including E43, might work cooperatively to assist the vertical movement of helix S4. These results imply that the range of the vertical mobility of helix S4 should be more suppressed in ion channels that are activated at higher membrane potentials (Fig. 6). Although the charged residues in the voltage sensor domain are generally well conserved, the residues at the positions corresponding to those of E43 and D60 in NaChBac are less conserved (36). The diversity in amino acids at these positions might thus contribute to differences in the voltage dependence of activation among voltage-gated ion channels.

Indications for the voltage-dependent movement of helix S4—Helix S4 has been proposed to underlie voltage-dependent activation by moving vertically in the lipid bilayer upon changes in membrane potential (18-20, 37). Movement of helix S4 is thought to result in a conformational change of the voltage sensor domain and eventually the opening of the pore gate (9). Double cysteine mutagenesis and structural studies of Kv8 showed that in the activated state the residues corresponding to T110 or R113 are close to helix S5 (12, 16), while in the resting state residues in the more extracellular part of helix S4 are close to residues in helix S5 (13). The D60K and E43K mutations increase the energy needed to activate the channels so that these two mutations keep the channel in the resting state as the disulfide bonds are formed. Therefore,
the change caused by the E43K or D60K mutations in the efficiency with which disulfide bonds are formed in QC, TC, and RC might indicate a shift in the residues that are close to each other during voltage-dependent gating (Fig. 5). Residue Q107, which is closer to the extracellular surface than residues T110 or R113, might be close to M164 in the resting state or in the early phase of voltage-dependent activation. These results imply that helix S4 moves vertically with Q107, T110, and R113 facing M164, and that the positive charges move from the intracellular towards the extracellular side during voltage-dependent gating. The voltage-dependent movement of helix S4 in NaChBac suggested by our double cysteine mutagenesis and additional lysine substitution of residues E43 and D60 is consistent with previously proposed models for voltage-dependent gating. For example, the helical screw and paddle models both require a large movement of helix S4 along its axis (20, 37). It was recently shown that the voltage sensor of NaChBac has an overall structure similar to Kv1s and changes its conformation during activation in the same manner as well (38). The rotational and vertical movement of helix S4 in NaChBac indicated by our cysteine mutagenesis agrees well with the helical screw model, which requires that helix S4 move both vertically and rotationally. Our results thus indicate that the movement of helix S4 in NaChBac is similar to that of canonical voltage-gated ion channels. Our study only shows, however, that helix S4 is mobile. Structural studies are needed to reveal its detailed movement during voltage-dependent activation of NaChBac.

REFERENCES


**FOOTNOTES**

The abbreviations used are: Na<sub>V</sub>, voltage-gated Na channel; K<sub>V</sub>, voltage-gated K channel; QC, Q107C/R113A/M164C mutant; TC, T110C/R113A/M164C mutant; RC, R113C/M164C mutant.

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**FIGURES AND FIGURE LEGENDS**

**Fig. 1** Amino acid sequence and predicted topology of NaChBac. (A) Alignment of the sequence of NaChBac with some K<sub>V</sub>s obtained with clustalW 1.81. NaChBac: Na<sub>V</sub> from *Bacillus halodurans* C-125 (GI: 10174118), KvAP: K<sub>V</sub> from *Aeropyrum pernix* K1 (GI: 5104624), K<sub>V</sub>1.2/2.1: α-subunit of the chimeric K<sub>V</sub>1.2/K<sub>V</sub>2.1 channel (GI: 160877792), Shaker: α-subunit of the Shaker K<sub>V</sub> from *Drosophila melanogaster* (GI: 288442). The residue numbers at the top and bottom are based on the sequences of NaChBac and Shaker, respectively. The arrowheads indicate residues reported to be close between helices S4 and S5 in the Shaker channel, R362, F416, and A419 (12), and the brown lines mark the residues mutated to cysteine in the present study, A106 to V117 in S4 and V160 to E172 in S5. (B) Putative transmembrane topology of NaChBac. Helices S1-S4 form the voltage sensor domain and helices S5-S6 form the pore domain. The elliptical boxes indicate residues Q107, T110, and R113 in S4 and M164 in S5, which were particularly efficient in forming inter-subunit disulfide bonds. The rectangular boxes indicate residues E43 and D60 that were mutated to lysine. (C) Putative domain arrangement in a Na<sub>V</sub> tetramer...
with each monomer shown in a different color. The four pore domains form an ion conducting pore at the center of the channel, and each voltage sensor domain is located near the pore domain of the adjacent subunit.

**Fig. 2** Identification of paired cysteine mutations in helices S4 and S5 that lead to the formation of inter-subunit disulfide bonds. NaChBac mutants with a cysteine substitution of a residue each at the extracellular sides of helix S4 and helix S5 were analyzed by Western blotting for inter-subunit disulfide bond formation. Gels were run under non-reducing conditions. The numbered arrows mark bands representing monomer, dimer, trimer, and tetramer species. (A) The R113C substitution in helix S4 was combined with a series of substitutions of residues at the extracellular side of helix S5 (from V160 to E172). The reason why most disulfide-bonded tetramers appear as doublet bands is the differential migration of tetramers with three disulfide bonds, which will migrate as a linear polypeptide, and tetramers with four disulfide bonds, which migrate as a circular polypeptide. The difference in migration of tetramers with three or four disulfide bonds was previously observed in the studies on the Shaker channel (39). (B) The M164C substitution in helix S5 was combined with a series of substitutions of residues at the extracellular side of helix S4 (from A106 to V117).

**Fig. 3** Inter-subunit disulfide bonds formed in NaChBac T110C/M164C and R113C/M164C mutants lock the channels in a non-conductive state. Representative current traces for wild-type (A) and R113C/M164C mutant (B) generated by a series of test-pulses in HEK-293 cells. Currents were recorded without (top) or with (bottom) a 30-min preincubation with 5 mM DTT. The panels below each current trace are averaged I-V curves derived from each experimental condition. Error bars correspond to the S.E. of the mean.

**Fig. 4** The D60K and E43K mutations shift the activation of NaChBac to a more positive membrane potential. Normalized curves of the voltage-dependent activation of NaChBac mutants derived from CHO-K1 cells. The voltage dependence was determined by measuring deactivation tail currents. Error bars correspond to the S.E. of the mean. (A) Activation curves for wild-type NaChBac (closed circles), and the E43K (open circles) and D60K (closed triangles) mutants. (B) Activation curves for the R113A single mutant (closed circles), and the E43K/R113A (open circles) and D60K/R113A (closed triangles) double mutants.

**Fig. 5** The D60K and E43K mutations change which residues in the double-cysteine mutants
preferentially form disulfide bonds. Residues D60 or E43 were mutated to lysine in the
Q107C/R113A/M164C (QC), T110C/R113A/M164C (TC), and R113C/M164C (RC) mutants. Membranes isolated from *E. coli* expressing these mutants were analyzed by quantitative Western blotting. (A) Representative Western blot of NaChBac double cysteine mutants with no additional mutation (left panel), with the D60K mutation (middle panel), and the E43K mutation (right panel). The numbered arrows indicate the bands representing the monomer, dimer, trimer, and tetramer species. (B) Quantification of the amount of disulfide-bonded tetramer that formed with the various mutants. The presented values are means of the intensity of the tetramer band divided by the sum of the intensity of all protein bands (no addition: *n* = 9, D60K: *n* = 9, E43K: *n* = 6). Error bars correspond to the S.E. of the mean. Asterisks indicate the differences from control (*: *P* < 0.05, **: *P* < 0.01). Comparisons without asterisks were not statistically significant.

**Fig. 6** Schematic drawing of the relative position of helix S4 to helix S5 in NaChBac. Interface between helix S4 (blue) and helix S5 of the adjacent subunit (yellow). In the wild-type channel, helix S4 has significant vertical mobility (indicated by a long black arrow in the left panel), which is facilitated by ionic interactions between the negatively charged residues E43 or D60 with positive charges in helix S4. The E43K or D60K mutations introduce a positive charge, which repulses the positive charges in helix S4 and may thus restrict the mobility of helix S4 (indicated by the shorter red arrow in the right panel). As a result, higher membrane potentials are required to activate the E43K and D60K mutants (bottom panel).
TABLE 1
The time constants of NaChBac cysteine mutants

<table>
<thead>
<tr>
<th></th>
<th>(\tau_{\text{act.}})</th>
<th>(\tau_{\text{inact.}})</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>NR 6.11 ± 0.9</td>
<td>91.3 ± 11</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>R   4.70 ± 1.2</td>
<td>74.4 ± 5.3</td>
<td>6</td>
</tr>
<tr>
<td>R113C</td>
<td>NR 8.46 ± 1.1</td>
<td>141 ± 11</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>R   5.95 ± 0.7</td>
<td>122 ± 12</td>
<td>10</td>
</tr>
<tr>
<td>M164C</td>
<td>NR 12.7 ± 1.4</td>
<td>138 ± 8</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>R   10.6 ± 1.3</td>
<td>165 ± 28</td>
<td>6</td>
</tr>
<tr>
<td>R113C/M164C (RC)</td>
<td>NR ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R   11.2 ± 0.4</td>
<td>84.1 ± 11</td>
<td>7</td>
</tr>
</tbody>
</table>

Each mutant was expressed in HEK-293 cells. The time constant of activation (\(\tau_{\text{act.}}\)) is the time from 10% to 90% of the peak current. The time constant of inactivation (\(\tau_{\text{inact.}}\)) is the time from the peak current to \(1/e\) of the peak current. The \(\tau_{\text{act.}}\) and \(\tau_{\text{inact.}}\) were calculated at +10 mV membrane potential. All results are expressed as mean ± SE.

NR; non-reducing condition, R; reducing condition, ND; not detectable
<table>
<thead>
<tr>
<th></th>
<th>(\tau_{\text{act.}}) (ms)</th>
<th>(\tau_{\text{inact.}}) (ms)</th>
<th>(V_{1/2}) (mV)</th>
<th>(\kappa) (mV/e-fold)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>4.56 ± 0.7</td>
<td>86.1 ± 0.7</td>
<td>-36.6 ± 2.0</td>
<td>10.4 ± 1.6</td>
<td>7</td>
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<tr>
<td>E43K</td>
<td>3.30 ± 0.5</td>
<td>114 ± 20</td>
<td>18.0 ± 1.3</td>
<td>11.0 ± 1.0</td>
<td>6</td>
</tr>
<tr>
<td>D60K</td>
<td>ND</td>
<td>ND</td>
<td>42.0 ± 2.0</td>
<td>17.8 ± 1.5</td>
<td>6</td>
</tr>
<tr>
<td>R113A</td>
<td>9.02 ± 2.5</td>
<td>172 ± 13</td>
<td>2.59 ± 3.6</td>
<td>18.6 ± 2.3</td>
<td>10</td>
</tr>
<tr>
<td>E43K/R113A</td>
<td>ND</td>
<td>ND</td>
<td>24.5 ± 2.5</td>
<td>22.1 ± 1.9</td>
<td>6</td>
</tr>
<tr>
<td>D60K/R113A</td>
<td>ND</td>
<td>ND</td>
<td>80.3 ± 0.9</td>
<td>17.4 ± 0.7</td>
<td>6</td>
</tr>
</tbody>
</table>

Each mutant was expressed in CHO-K1 cells. The time constant of activation (\(\tau_{\text{act.}}\)) is the time from 10% to 90% of peak current. The time constant of inactivation (\(\tau_{\text{inact.}}\)) is the time from the peak current to 1/e of the peak current. The \(\tau_{\text{act.}}\) and \(\tau_{\text{inact.}}\) of WT were calculated at +10 mV membrane potential, and those of E43K, D60K, and R113A were calculated at +60 mV membrane potential. \(V_{1/2}\) of activation is the potential of 50% activation. \(\kappa\) is the slope factor. All results are expressed as mean ± SE. R; reducing condition, ND; not determined.
Figure 3

A  
$V_{\text{total}} = -120\text{mV}$  
$V_{\text{total}} = -90\text{mV}$  

WT  

B  
$V_{\text{total}} = -120\text{mV}$  
$V_{\text{total}} = -90\text{mV}$  

R113C/M164C  

---

WT + 5mM DTT  

R113C/M164C  
+ 5mM DTT  

---

$V_m (\text{mV})$  
Normalized Amplitude  

$V_m (\text{mV})$  
Normalized Amplitude  

---

1 nA  
100 ms  

1 nA  
100 ms  

0.5 nA  
100 ms
Figure 4

A

B

-100  -50   0  50  100
Membrane Voltage (mV)

0.0  0.2  0.4  0.6  0.8  1.0
Normalized Current

 wild-type ($V_m = -50$ mV)
 D43K ($V_m = -10$ mV)
 D50K ($V_m = -40$ mV)

 D113A ($V_m = -5$ mV)
 E43K/R113A ($V_m = 25$ mV)
 D50K/R113A ($V_m = 80$ mV)
Figure 5

A

B

Tetramer : total ratio (%)

Additional mutation

No D60K E43K

QC TC RC QC TC RC QC TC RC
Figure 6
Arrangement and mobility of the voltage sensor domain in prokaryotic voltage-gated sodium channels
Takushi Shimomura, Katsumasa Irie, Hitoshi Nagura, Tomoya Imai and Yoshinori Fujiyoshi

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