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Inferred motions of the S3a helix during voltage-dependent K⁺ channel gating

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Abstract

The gating of voltage-dependent potassium channels is controlled by conformational changes in voltage sensor domains. Previous studies have shown that the S1 and the S2 helices of the voltage sensor are static with respect to motion across the membrane, while the voltage sensor paddle consisting of the C-terminal half of S3 (S3b) and the charge-bearing S4, is mobile. The mobile component is attached to S1 and S2 via the S2-S3 turn and the N-terminal half of S3 (S3a). In this study we analyze KvAP, an archaebacterial voltage-dependent potassium channel, to study the mobility with respect to translation across the membrane of S3a. We utilize an assay based on attachment of tethered biotin and its site-specific accessibility to avidin. Our results reveal that the S3a helix does not move appreciably across the membrane in association with gating. The static behavior of S3a constrains the conformations available to the voltage sensor when it closes and suggests that a set of negative counter charges within the membrane's inner leaflet remains intact in the closed conformation.

Keywords

potassium channel; voltage-dependent gating; voltage sensor; membrane protein; electrical signaling

Introduction

Cell membranes function as electrical capacitors capable of storing a charge separation between inside and out. When a K⁺ channel opens, K⁺ flows from inside the cell where the K⁺ concentration is higher to the outside where the concentration is lower. The net outward movement of K⁺ charges the membrane negative on its inner surface relative to the outside. When Na⁺ channels open, the opposite occurs: Na⁺ flows inward from its higher concentration outside, causing the inside to charge positive. In this manner ionic gradients and selective ion channels charge the cell membrane capacitor.¹ The magnitude of the ionic gradients typically found across living cell membranes gives rise to voltage differences of approximately 100 mV. Given the thickness of a cell membrane, approximately 30 Å for the hydrophobic core, voltages of this magnitude result in an electric field within the membrane greater than 10⁷ volts per meter. In special circumstances, nature uses this strong electric field to control the conformation

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of membrane-embedded proteins to regulate their function. A dramatic example of this property is exhibited by voltage sensors, which are membrane proteins that have evolved specifically to undergo voltage-dependent conformational changes.² Voltage sensors are best known as domains that regulate the gating of voltage-dependent ion channels.

The atomic structures of voltage sensor domains from two different voltage-dependent K⁺ (Kv) channels have been determined by X-ray crystallography. One of these is from a prokaryotic Kv channel named KvAP and the other from a eukaryotic Kv channel, Kv1.2, and its variant named paddle chimera (Fig. 1).³⁻⁵ Despite their different origins, the two voltage sensor structures are very similar. They consist of four mainly helical membrane crossings referred to as S1-S4. S4 contains a basic amino acid (positive charged, usually arginine) every third amino acid. By exerting a force on these charges the membrane electric field performs electromechanical work to influence the conformation of the voltage sensor.

The paddle chimera structure provides a clear picture of a voltage sensor in the context of a bilayer-like arrangement of lipid molecules.⁵ In this case, the K⁺ channel is open and the sensor is in a conformation that brings positive charged amino acids on S4 near the extracellular membrane surface, where we expect to find them in an open conformation voltage sensor.⁶⁻⁸ The structure of a closed conformation voltage sensor has not yet been determined, however, voltage sensor motions associated with membrane voltage changes have been inferred using state-dependent accessibility of cysteine residues to thiol reactive agents and state-dependent antibody binding.^{9,10}

One accessibility method, which is to be the main method used in this study and is described in detail below, employs avidin capture of biotin tethered to specific sites on the channel (Fig. 2a).^{9,11} Since avidin is a large macromolecule that cannot gain access to relatively narrow aqueous crevices in the protein, capture can occur only if the residue is positioned within a defined distance (depending on the tether length) from the membrane-water interface (Figs. 2b,c). Applied to KvAP, the biotin method showed that in association with gating, S1, S2, S5 and S6 remain relatively static in the membrane with respect to motion along a direction normal to the membrane plane (Z-axis). By contrast, a segment of S4 appears to change its position along the Z-axis by around 15 Å. These observations, taken together with crystal structures showing the close apposition of the C-terminal half of S3 (S3b) and S4 (forming a structure called the voltage sensor paddle), suggests that the voltage sensor paddle moves relative to S1, S2, and the pore (Figs. 1b,c).

The region of KvAP corresponding to the S2-S3 turn and the N-terminal half of S3 (S3a) was not studied using the biotin method. Structurally this region attaches the voltage sensor paddle to the S1-S2 half of the voltage sensor (Figs. 1 b, c). It is therefore relevant to our understanding of voltage sensor motions to know whether S3a remains static or moves with respect to the Z-axis when the channel gates. That is the question addressed in this study.

Results

In the biotin-avidin assay, cysteine mutations are introduced into a version of the channel in which the lone native cysteine was mutated to serine. Mutants were overexpressed, purified to homogeneity in the presence of detergents, and then conjugated separately to cysteine reactive linkers of various lengths with a biotin moiety attached to their ends (see Fig. 2c for the chemical structures of linkers used in this study). Conjugation was confirmed using a gel-shift assay in which the biotin-conjugated channel was exposed to avidin and then run on non-reducing SDS-PAGE (Suppl. Fig. S1). The conjugated mutant channels were reconstituted into lipid vesicles and then incorporated into a planar bilayer system in which the voltage-gated ion conduction properties were studied before and after the addition of avidin. In separate experiments, avidin

was added to the intracellular or extracellular side of the membrane, defined with respect to the physiological orientation of the channels in the membrane. Since the channels undergo repeated opening and closing steps during the assay, the measured accessibility of biotin determines the cumulative accessibility over all functionally relevant states sampled during the open-closed transition of the channel molecules. In all cases but one, the signature of avidin binding is depletion of the current when the membrane is depolarized. In other words, when we observe a decrease in the current, we conclude that avidin has accessed the biotin. Inhibition is not always complete and is frequently associated with changes in other characteristics of voltage-gated K^+ conduction. However, for the purposes of this study, we are only interested in the binary result of accessibility or inaccessibility of the biotin moiety to the added avidin. In control experiments we have demonstrated that in one representative case, reduction of the disulfide linkage attaching the biotin tether and the cysteine residue results in loss of susceptibility to avidin block of ion conduction. Also, *in situ* reduction of the disulfide can reverse avidin block of ion conduction. (Suppl. Fig. S2 and Supplementary Methods)

In the two previous studies, residue positions in S1, S2, S3b and S4 of the voltage sensor and S5 and S6 of the pore were evaluated.^{9,11} Here we focus on six positions shown as green spheres mapped onto KvAP (Fig. 1b) and the equivalent positions of the paddle chimera channel (Fig. 1c). The intracellular most position, A84C, conjugated with the 1.0 Å PDTE linker, was inhibited by addition of intracellular avidin (Fig. 3a, left) but not when avidin was added to the extracellular solution (Fig. 3a, right). Similar results were obtained with A84C conjugated to the 10 Å linker (Fig. 3b). For the 17 Å linker, avidin when added from either side (Fig. 3c, left) did not produce any measurable change in the voltage-activated current. This is the first and only case in which we have observed the disappearance of avidin's effect with lengthening of a tether. It seems possible that avidin binds in this case from the inside without influencing function because the 17 Å linker is sufficiently long and located at the aqueous surface (biotin on a 1.0 Å tether is accessed here) and outermost perimeter of the channel. Taken together, the data with different tether lengths at position 84 place this site within 1.0 Å of the intracellular solution and greater than 17 Å from the extracellular solution. To reemphasize the point that the biotin assay determines the cumulative accessibility over all functionally relevant states of the channel sampled during the open-closed transition, these results indicate that position 84 is within 1.0 Å of the intracellular solution at some time (if not all the time) and is greater than 17 Å away from the extracellular solution all the time.

Moving in the direction away from the intracellular solution and in towards the membrane, the next residue examined was K88 (Fig. 2). When functionalized with biotin on the 1.0 Å PDTE linker, avidin inhibited channel activity from the intracellular side (Fig. 4a, left) but not from the extracellular side (Fig. 4a, right). Both the 10 Å and the 17 Å linkers showed accessibility to avidin from the intracellular side as well (Fig. 4b, left and 4c, left) but not from the extracellular side (Fig. 4b, right and Fig. 4c, right). Thus, position 88 falls within the same region as position 84, very near the intracellular solution.

Based on the crystal structures, the next position, L91, is expected to lie within the membrane's hydrophobic core. Consistent with this expectation, avidin from the intracellular solution failed to inhibit the 1.0 Å conjugate, implying that the site is greater than 1.0 Å away from the intracellular solution (Fig. 5a, left). Lengthening the tether to 17 Å permitted inhibition from the intracellular side (Fig. 5b, left). Both the 1.0 Å and 17 Å conjugates were inaccessible from the extracellular solution (Figs. 5a, b, right). Measurements on L91C conjugated with the 10 Å IPEO linker were complicated by a "run-down" phenomenon in which current slowly decreased over time even in the absence of avidin when held at a negative holding voltage and repeatedly pulsed at fixed intervals to a constant positive voltage step. An example of this effect is shown in Fig. 5c. Even with this background current decay, inhibition by avidin from the intracellular side was still discernable as an immediate decrease in the current following

addition of avidin (Fig. 5d). This result, together with data from the 1.0 Å and 17 Å conjugates, places position 91 greater than 1.0 Å and less than 10 Å away from the intracellular solution and greater than 17 Å away from the extracellular solution.

The next positions evaluated – 94, 96 and 98 – were extremely difficult to study due to a combination of “run-down” phenomenon and few functional channels per membrane. Nevertheless we were able to obtain membranes reproducibly that contained at least a few stable voltage-dependent channels. These membranes yielded clearly interpretable data for a single linker length on each of these three positions. Channels with a 10 Å IPEO linker conjugated at position 94 were not affected by addition of avidin to either side of the membrane, suggesting that this position is greater than 10 Å from either membrane surface at all times during the gating transition (Fig. 6a). Channels with a 17 Å BCAC linker conjugated at position 96 were inhibited by avidin from the intracellular solution (Fig. 6a, left) but not from the extracellular solution (Fig. 6b, right). Channels with a 10 Å IPEO linker conjugated at position 98 were not inhibited from the extracellular solution (Fig. 6c). Because biotin conjugated with the 10 Å linker to position 94 is inaccessible from the internal solution (Fig. 6a, left), internal avidin was not tested with this linker at position 98.

Discussion

We have now scanned every membrane-spanning region of the KvAP K⁺ channel using the biotin-avidin method. It is noteworthy that the S2-S3 turn and S3a helical segment is the least tolerant region in terms of achieving analyzable voltage-dependent channels with a tethered biotin. In other regions of the channel, conjugation of biotin influenced the midpoint of the Boltzmann activation curve and the kinetics of activation, deactivation, and inactivation to variable extents. However, it was still possible to close and open the channels with voltage steps from very negative voltages (near –100 mV) to very positive voltages (near 100 mV). In S3a not all tether lengths could be studied, but the data are sufficient to address the question we set out to answer: does the S3a segment move on the Z-axis with gating?

Fig. 7 shows the new data acquired in this study mapped onto an atomic structure together with data from previous studies.^{5,9,11} The biotin accessibility data were all obtained using KvAP in planar lipid membranes.^{9,11} The structural model is that of the paddle chimera channel.⁵ The sequence alignment correlating amino acid positions in KvAP and the paddle chimera channel is given in Fig. 1a. Data are presented for each tether length separately: black spheres show positions inaccessible from either side of the membrane while blue, red, and yellow spheres show positions accessible from the intracellular, extracellular, and both solutions, respectively. Planar surfaces on the stereo images indicate the demarcations between accessible and inaccessible positions as defined by the previously determined static (with respect to the Z-axis) helical components S1, S2, S5 and S6. It is evident that the newly mapped sites in the S2-S3 turn and S3a helix exhibit the same accessibility pattern as the static components. We therefore conclude that S3a does not undergo significant Z-axis translational movements when the voltage sensor undergoes its voltage-dependent conformational changes. This finding is consistent with the idea that the S2-S3 turn at least in part serves as an anchor at the intracellular membrane-water interface.¹² The sensitivity of this region of the channel to perturbation (i.e. biotin conjugation) implies that it might undergo some degree of structural rearrangement in association with gating, however, the motions appear not to be in the direction of the Z-axis.

The conclusion that S3a does not change its depth within the membrane during gating has two implications for voltage sensor conformational changes. The first implication is that the large Z-axis translation of S4 (see outlying accessibility pattern on S4 in Fig. 7) does not come about through slippage or translation of S3a relative to S1 and S2. In the absence of S3a Z-axis motion, 15 Å translations of S4 would require either slack in the loop connecting S3 to S4 or a break

in the S3 helix so that S3b can move when the channel gates. KvAP has no loop between S3 and S4 and experiments with the Shaker K⁺ channel show that it functions quite normally when the S3-S4 loop is deleted.¹³ Hence motion of S4 cannot be structurally accommodated merely by conformational change in the loop preceding S4.

Certain aspects of the voltage sensor structure support the idea of a break or hinge-like motion within S3, between S3a and S3b. First, sequence alignment of voltage gated potassium channels reveal a conserved proline residue in S3 that demarcates S3a and S3b. The proline, by disrupting the normal hydrogen bonding pattern of an α -helix, might serve to introduce a weak point, conserved through evolution, at which S3 can break under force. Second, because S4 and S3 are physically juxtaposed next to each other, even a minimalist model invoking large Z-axis motion of S4 necessarily contradicts a completely static S3b. And third, in different structures of KvAP, S3b and S4 are associated with each other as a packed unit^{3,12}, suggesting that they move as a packed unit. For these reasons we think that the occurrence of a break in S3, between S3a and S3b, seems likely when the voltage sensor changes from its open conformation (observed in the crystal structure, Fig. 7) to its closed conformation. A comparison of the crystal structures of the KvAP isolated voltage sensor and the paddle chimera voltage sensor offer a suggestion as to how such a break might occur during gating (Fig. 8a). In the paddle chimera channel, the S3 helix axis is bent approximately 30° at the junction between S3a and S3b. In KvAP the helix is unwound at the junction and S3b adopts a very different angle (than is seen in the paddle chimera voltage sensor) with respect to S3a. The biotin accessibility of S3b suggests that it remains within the extracellular leaflet of the membrane bilayer even in the closed conformation (Fig. 7). A rotational motion centered about an axis near the S3a-S3b demarcation would permit S3b to remain in the extracellular leaflet while enabling S4 residues to move across the membrane and account for the pattern of biotin accessibility. The biotin accessibility pattern together with the crystal structures of paddle chimera and KvAP lead us to the general conclusion depicted in figure 8b, which shows in grey static elements of the voltage sensor (S1, S2 and S3a) and in red mobile elements (S3b, S4 and the S4-S5 linker). Such motion of the S3b-S4 voltage sensor paddle could impart force to gate the pore via the S4-S5 linker.

The second implication of S3a maintaining a constant depth within the membrane is that the environment created by S1, S2 and S3a observed in the paddle chimera crystal structure (an open voltage-dependent K⁺ channel) is likely to be preserved in the closed conformation (Fig. 8b) of the channel.⁵ This environment provides acidic amino acids from S2 (E236), S3a (D259) and S0 (E154), which form the internal negative cluster that stabilize K302 and R305 in the open conformation. Preservation of this environment in the closed conformation would leave the internal negative cluster intact to stabilize the outermost S4 arginine residues as they cross the phenylalanine gap to the inside of the membrane upon voltage sensor closure.

Experimental Procedures

Preparation of biotinylated channels

The single native cysteine, C247, was mutated to C247S to yield a version of KvAP without any cysteine. Cysteine mutations were introduced on this background using the QuikChange method (Stratagene) and the mutations verified by sequencing through the entire gene. Mutant channels were expressed, purified and functionalized with PDTE biotin, IPEO biotin or BCAC biotin as described^{11,14}. Biotinylated channels were reconstituted into lipid vesicles as described elsewhere¹⁵.

Electrophysiology

Electrophysiology of biotinylated channels was carried out as described.¹⁴ Biotinylated channels were incorporated into planar membranes and studied using various voltage protocols before or after the addition membrane. For most biotinylated channels, the effect of avidin binding on channel function was tested by 200 ms depolarizing pulses to +120 mV from a holding voltage of −120 mV every 120 s. Some biotinylated channels, due to a shift in voltage dependent activation, required more negative holding voltages (up to −160 mV).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1.
 Residues tested in this study. a) Sequence alignment of the Kv1.2-2.1 chimera (paddle chimera), KvAP (GI, 5104624), rat Kv2.1 (GI, 24418849), rat Kv1.2 (GI, 1235594), Shaker Kv (GI, 13432103) and KcsA (GI, 61226909). Secondary structure elements are indicated above the sequences. Selected conserved residues are shown in red (negatively charged), blue (positively charged) and green (hydrophobic). The residues tested in this study and the corresponding residues in the chimera are highlighted in orange. b) Stereo view of a subunit of the isolated KvAP voltage sensor (grey α -carbon trace, PDB accession code, 1ORS). The α -carbon atoms of the residues tested in this study are shown as green spheres. c) Stereo view showing a subunit of the paddle chimera (grey α -carbon trace, PDB accession code, 2R9R).

The α -carbon atoms of residues tested in this study have been mapped onto the structure using the alignment in a) and shown as green spheres.

Fig. 2.

Biotin-avidin capture assay. a) Avidin being a large protein cannot penetrate into small aqueous crevices (left) and can only bind biotin when the tether length is sufficient to allow the presentation of biotin beyond a defined distance outside of the membrane – aqueous border (right). b) The pore regions from two subunits of KvAP are shown from the side. A biotin conjugated to a Cys residue in the protein can only bind avidin (shown as $C\alpha$ trace in blue) if the $C\alpha$ of the Cys lies within the effective tether length of the membrane-aqueous border (shown as black line). For the specific tether, this distance defines the zone of accessibility. c) Detailed chemical structures of the tethered biotin reagents used in this study. Upon avidin binding,

biotin and 7 Å of the tether (shown in red) become buried. The remainder of the tether defines the effective tether length, which is shown on top of each tether.

Fig. 3. Representative experiments showing the effect of avidin binding to A84C KvAP conjugated to tethers of different lengths. Current traces were elicited after depolarization to positive voltages before (black) or after the addition of internal (blue) or external (red) avidin. a) 1.0 Å PDTE linker (n=3, internal; n=2, external). b) 10 Å IPEO linker (n=2, internal and external) and c) 17 Å BCAC linker (n=3, internal; n=2, external). Current traces shown are average traces of at least four traces.

Fig. 4.

Representative experiments showing the effect of avidin binding to K88C KvAP conjugated to tethers of different lengths. Current traces were elicited after depolarization to positive voltages before (black) or after the addition of internal (blue) or external (red) avidin. a) 1.0 Å PDTE linker (n=2, internal and external). b) 10 Å IPEO linker (n=2, internal and external) and c) 17 Å BCAC linker (n=2, internal and external). Current traces shown are average traces of at least four traces.

Fig. 5.

Representative experiments showing the effect of avidin binding to L91C KvAP conjugated to tethers of different lengths. Current traces were elicited after depolarization to positive voltages before (black) or after the addition of internal (blue) or external (red) avidin. a) 1.0 Å PDTE linker ($n=5$, internal and external). b) 17 Å BCAC linker ($n=3$, internal; $n=2$, external). Current traces shown are average traces of at least four traces. c) and d) L91C conjugated to the 10 Å IPEO linker. c) Individual current traces elicited by depolarization to the same positive voltage step repetitively at the same fixed interval (left). Graph showing the mean current elicited at each pulse between fixed time intervals within the pulse as a function of time (right). No avidin was added ($n=2$). d) Individual traces showing currents elicited after depolarization

to the same positive voltage before (black) and after (blue) the addition of avidin to the internal side ($n=2$).

Fig. 6.

Representative experiments showing the effect of avidin binding to different mutants of KvAP conjugated to biotin linkers. Individual traces elicited after depolarizing to a constant positive voltage step at fixed intervals are shown before (black) and after addition of internal (blue) or external (red) avidin. a) I94C conjugated to the 10 Å IPEO linker (n=2, internal and external). b) A96C conjugated to the 17 Å BCAC linker (n=3, internal and external). c) V98C conjugated to the 10 Å IPEO linker (n=3).

Kv1.2-2.1 Chimera Structure

1 Å PDTE

10 Å IPEO

17 Å BCAC

Fig. 7.

Summary of the biotin-avidin accessibility data. Stereo view of a single subunit of the paddle chimera with accessibility data from the previous study¹¹ and the current study mapped onto it using the alignment in Fig. 1a. Amino acids are represented by their α -carbon atoms shown as a sphere. Red residues are accessible only to external avidin, blue residues are accessible only to internal avidin, black residues are inaccessible, and yellow residues are accessible to avidin from both sides. Horizontal planes demarcate between regions of accessibility and inaccessibility. For the 17 Å BCAC linker, a single plane separates internal and external accessibilities.

Fig. 8. Implications about conformational changes of the voltage sensor. a) Superposition of the S3 and S4 from the voltage sensors of the isolated voltage sensor of KvAP (PDB accession code, 1ORS) shown in green α -carbon ribbon trace and the voltage sensor from the paddle chimera (PDB accession code, 2R9R) shown in cyan α -carbon ribbon trace. The superposition was done using main chain atoms of residues 90-97 in KvAP and residues 256-263 in paddle chimera. b) Stereo view of the voltage sensor and part of the S4-S5 linker of paddle chimera (PDB accession code, 2R9R). The view is from the side. The static elements are shown in grey α -carbon trace and labeled in grey. The mobile elements are shown in red α -carbon trace and labeled in red. Selected residues are shown in ball and stick representation and are colored as follows – red, carbon (mobile elements); grey, carbon (static elements); blue, nitrogen; red, oxygen; green, phenylalanine 233; cyan, water. Ionized hydrogen-bonds between acidic and basic residues are shown with dashed lines.