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The influence of lipids on voltage-gated ion channels

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Voltage-gated ion channels are responsible for transmitting electrochemical signals in both excitable and non-excitable cells. Structural studies of voltage-gated potassium and sodium channels by X-ray crystallography have revealed atomic details on their voltage-sensor domains (VSDs) and pore domains, and were put in context of disparate mechanistic views on the voltage-driven conformational changes in these proteins. Functional investigation of voltage-gated channels in membranes, however, showcased a mechanism of lipid-dependent gating for voltage-gated channels, suggesting that the lipids play an indispensible and critical role in the proper gating of many of these channels. Structure determination of membrane-embedded voltage-gated ion channels appears to be the next frontier in fully addressing the mechanism by which the VSDs control channel opening. Currently electron crystallography is the only structural biology method in which a membrane protein of interest is crystallized within a complete lipid-bilayer mimicking the native environment of a biological membrane. At a sufficiently high resolution, an electron crystallographic structure could reveal lipids, the channel and their mutual interactions at the atomic level. Electron crystallography is therefore a promising avenue toward understanding how lipids modulate channel activation through close association with the VSDs.

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Introduction

The superfamily of voltage-gated ion channels consists of integral membrane proteins that contain four voltage-sensor domains (VSDs) and a central ion-conducting pore domain [1,2]. Members of this superfamily have been identified in all cells, and play critical roles in a variety of cellular physiology, from muscle contraction to neuronal activity to T cell activation in inflammatory (immune) response. Voltage-gated ion channels are divided into two broad groups: the hyperpolarization-activated and the depolarization-activated channels. Biophysical studies have shown that the VSDs in these two groups work in a similar way [3]. In both cases, the VSDs undergo significant conformational changes driven by electrical energy. These conformational changes are coupled to the pore domain, to close or open the ion channel in response to electrical stimuli [3–6]. The hyperpolarization-driven state of the VSD is called the 'DOWN' conformation (also resting or closed), and the depolarization-stabilized state is named the 'UP' conformation (also activated or open) [7••]. Understanding the structural basis for the voltage sensor function in membranes not only is fundamentally important for revealing the exquisite electrical control of protein structure, but also will forge the foundation for developing new therapeutical strategy for human diseases caused by the dysfunction of these channels [8,9].

All known VSDs are made of four helical transmembrane segments (S1–S4) with highly conserved charged residues on the second (S2) and fourth (S4) helices. During voltage-dependent gating, the charged residues on S4 translocate from one side of the transmembrane electric field to the other while the VSDs switch their conformations and couple the charge movement to the opening and closing of the channel pore [6,10,11,12]. Within each VSD there are water-accessible crevices from either side of the membrane [13]. The transmembrane electric field penetrates into these crevices to establish a certain degree of electric focusing [14]. In the UP conformation the gating charges (mainly on S4) are in the extracellular crevice and in the DOWN conformation in the intracellular one. Switching between the UP and DOWN conformations requires a significant energy input from the electric field, ~7.5 kcal/mol per VSD [15–18].

While a number of different structures of voltage-gated ion channels have been determined it remains unclear how the VSDs couple the charge movement to the pore opening and closing [6]. Three different groups of mechanistic models have been proposed and experimentally supported: first, the voltage sensor paddle model; second, the transporter-like model; third, the helical-translocation/helical-screw model. The voltage sensor paddle model argues for a 15–20 Å motion of the paddle (the helix-loop-helix motif composed of the S3b, the S3S4 linker and the extracellular half of S4) along the membrane normal [19•,20]. It does not exclude lateral motion or rotation of the S4, nor does it specify how the other

parts of the VSD adjust to accommodate the major structural changes in membranes. The transporter-like model stemmed from intramolecular distance measurements, and argues that the toggling of the fixed gating charges from the outward-facing to the inward-facing state needs a small-scale (4–6 Å or less) vertical movement of S4, traversing a narrow hydrophobic septum (plug) in the gating pore [21•,22•,23]. The transmembrane electric field is thought to be highly focused across such a short distance [14,18]. The third group of models proposed a vertical displacement of the S4 inside the gating pore with varying distances, and the helical screw model adds a ~180° rotation of S4 in order to reorient the charged residues on S4 [17,24].

Besides the uncertainty on the VSD's mode of action, there is mounting evidence that lipids influence the structural stability and function of the VSDs and therefore the opening and closing of the channel pore. Functional studies of voltage-gated channels in membranes highlight a lipid-dependent gating mechanism. Studies indicate that without any change in transmembrane voltage, manipulating the lipid composition in a membrane switches the VSDs between the DOWN and UP conformations [7••]. This and other studies suggest that the lipids exert strong gating effects on the voltage-gated channels [7••,25••,26••,27••].

In this review we highlight some of the key structural features of voltage-gated ion channels and discuss how lipids were shown to influence channel structure and function. We then highlight electron crystallography as a structural biology technique that could provide information about how the lipids interact with the VSDs to affect channel gating.

An overview of voltage-gated ion channel structures

Structures of four channels that contain VSDs or VSD-like domains have been determined to date: KvAP, Kv1.2 (and its chimera), MlotiK and NavAb [28••,29••,30••,35••]. MlotiK is a ligand-gated channel with VSD-like domains, but has not been found to be functional yet. KvAP, Kv1.2 and NavAb are functional voltage-gated channels. The four structures confirm the common topology that was previously proposed for the superfamily of voltage-gated ion channel. The channels are tetrameric assemblies (Figure 1a,b). The first four helices in each monomer constitute the VSD, and the sequence between helices 5 (S5) and 6 (S6) forms the pore domain. The loop connecting S5 and S6 forms the ion selectivity filter. Four pore domains (S5S6 from each monomer) assemble together around the 4-fold axis to create an ion-conducting pore.

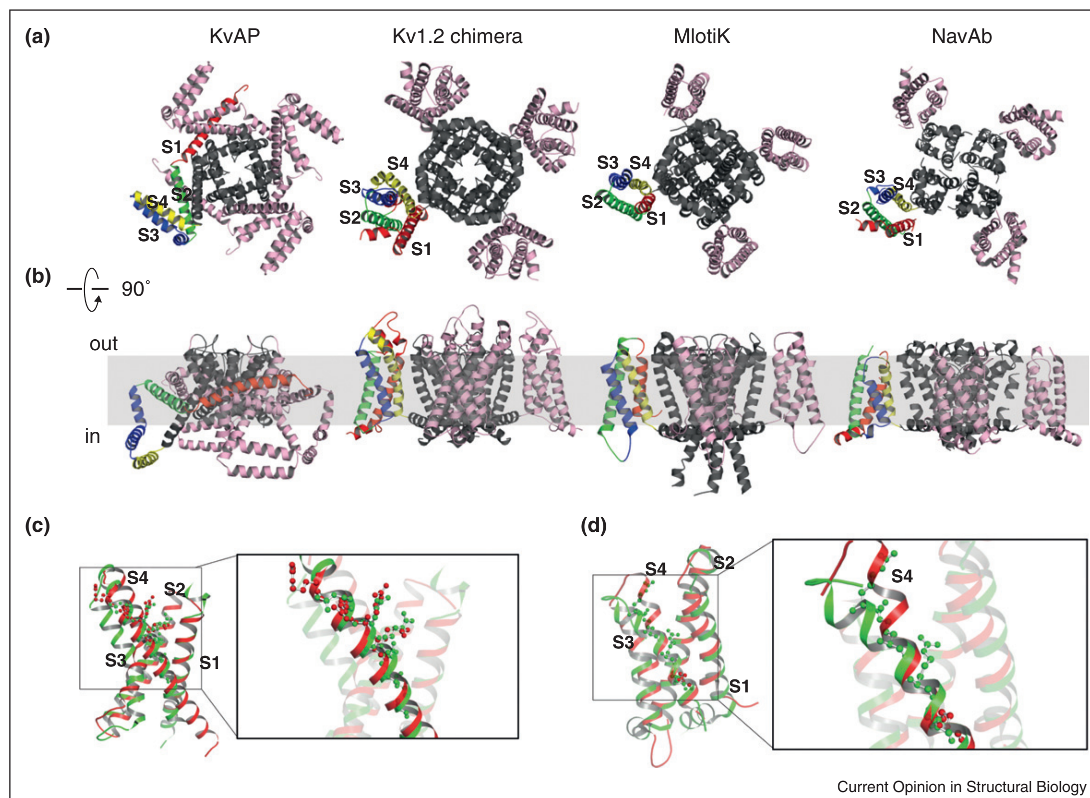
The conformation of the VSD from the full-length KvAP is significantly different from those in the Kv1.2 and its chimera, MlotiK and NavAb (Figure 1a,b). It is fully splayed with helices S1 and S2 wrapped along the side of the pore (Figure 1b, red and green helices). In other three cases the VSDs are folded into a compact 4-helix bundle neatly tucked to the lateral side of the pore (Figure 1a). The structure of the isolated KvAP VSD resembles closely that of the VSDs from Kv1.2 (as well as the Kv1.2/2.1 chimera; Figure 1c, overlay). S1, S2 and the top part of S4 overlay very well, but the position of S3b is different, displaying approx a 90° rotation between the two VSDs. Moreover, the positions of S4 arginines differ between KvAP and Kv1.2 as if they are shifted down by one register in the latter (Figure 1c), and the intracellular half of the S4 in the Kv1.2 chimera structure shows a short 3_{10} helix, which is absent in the same location of the other three VSDs.

The VSD structures of MlotiK and NavAb exhibit good overall fit among all four helices (Figure 1d). MlotiK has only one conserved charge in its S4. Its VSDs appear to be in a permissive 'UP' state, leaving the control of the channel pore to the intracellular nucleotide binding domains. In both channels, the N-terminal halves of their S4 segments harbor a short 3_{10} helix. Charged residues along the 3_{10} helix face the same side, which has implications for sliding the S4 across a newly named charge transfer center without much rotation [29••,30••,31•].

Although the conformational change that ensues in the VSDs in response to voltage is not clear, what is agreed upon is that the movement in the VSD helices is tightly coupled to the pore opening/closing. Exactly how the VSD and pore are coupled is not entirely clear. Two different coupling schemes were proposed for Kv1.2 and NavAb [28••,29••]. The first is based on the observed interaction between the S4–S5 linker and the intracellular half of S6. It was suggested that the sliding motion of S4 pushes the S4S5 linker intracellularly as well as the intracellular end of S6, leading to pore closure at a conserved PVP motif [28••]. This coupling scheme gained support from both structural and functional studies [32]. The second coupling scheme is based entirely on structural comparison between Kv1.2/2.1 chimera and NavAb, whose pore domains are in the open and closed states, respectively. It was suggested that wobbling the VSD could lead to a lateral rotation of the S4S5 linker, which in turn exerts a torque on the S5 and S6 to gate the pore with only a limited vertical movement of the S4. In previous biophysical analysis, the first closing step was found to bear weak voltage-dependence (0.5–1.0 e_0), which seemingly agrees with the small adjustment of the VSD to close the pore even though it is unclear what contributes to the small charge displacement [33,34].

While the available structures helped tremendously in understanding voltage-gated ion channels, there are still

Figure 1



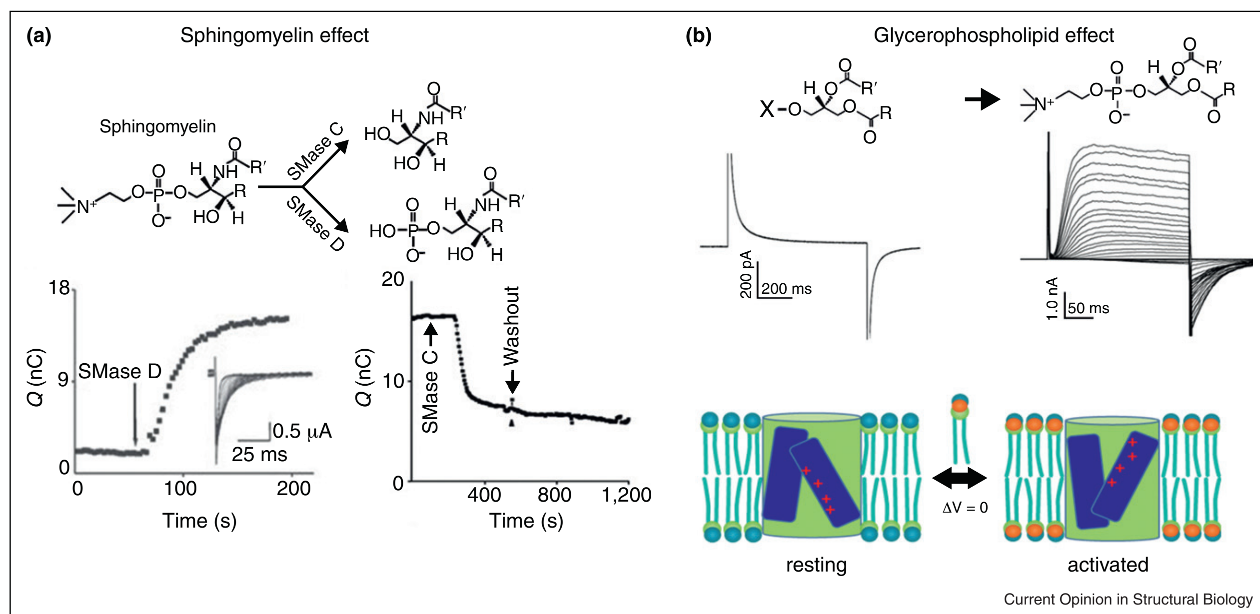
The X-ray structures of ion channels containing VSDs. **(a)** Top views of the structures of KvAP (PDB code 1ORS), Kv1.2/2.1 chimera (PDB code 2R9R), MlotiK (PDB code 3BEH) and NavAb (PDB code 3RW0). One voltage sensor domain in each channel is colored as S1 (red), S2 (green), S3 (blue) and S4 (yellow). **(b)** Side views of the four channels in a putative lipid bilayer (hashed gray). **(c)** Comparison of the voltage-sensors of KvAP (red) and Kv1.2 (green) with the side chains of the arginine residues presented as colored balls. **(d)** Comparison of the voltage-sensors of MlotiK (red) and NavAb (green) in the same view as in (c). Figures were produced using PYMOL.

many unanswered questions. The problem is further complicated by various unexplained effects observed on channels (pore and VSD) in the presence of various detergents and/or lipid molecules. For example, the structure of the VSD from KvAP varied in the presence of different detergents such as β-octyl-glucoside or diheptyl-phosphotidyl-choline and the presence or absence of long acyl chain detergents and lipid molecules used in various studies seem to affect the structural stability and function of the channels [28••,35••,36•]. It is also unclear how well the VSDs are coupled to the pore in the Kv1.2 and NavAb structures, even though the assignment of the conformations appears coherent with biophysics analysis [28••,29••].

The influence of lipids on voltage-gated ion channels

Voltage-gated ion channels function in membranes in which lipids associate closely with both the VSDs and pore domains. It is therefore not surprising that lipids can influence the structure and function of these channels. Recent studies suggest that changing lipid composition alone, without a change in transmembrane voltage, caused the VSDs to switch conformations [7••]. In oocytes expressing Kv2.1 and other *Shaker*-like channels, sphingomyelinase (SMase) D treatment was able to increase the total number of active channels [25]. The action of SMase C instead suppressed the total channel activity by decreasing the number of active channels [26] (Figure 2a). In other experiments, phospholipids were found to be required for KvAP to reach the open state, and lipids lacking phosphate headgroups (called nonphospholipids hereafter) were found to stabilize the VSDs in the 'DOWN' conformation [7••,27••] (Figure 2b). Comparison of the chemical nature of the products from SMase D/C treatment with the lipids that gate the KvAP channel (Figure 2a,b) suggests that ceramide molecules produced by SMase C catalysis exert lipid-dependent gating on the *Shaker*-like channels and favor the 'DOWN' conformation of the VSDs in these channels. SMase D treatment instead liberated ceramide-1-phosphates which favor the 'UP' conformation.

Figure 2



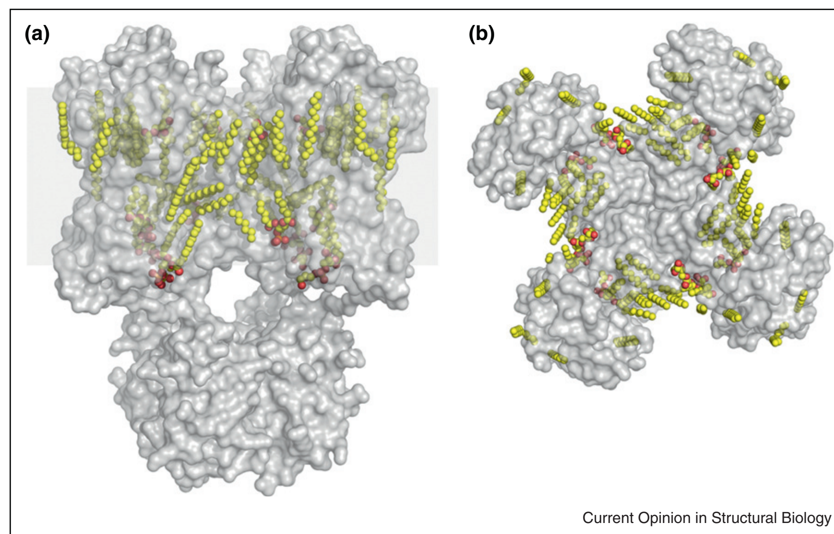
Lipid-dependent gating of voltage-gated ion channels. **(a)** Sphingomyelinase (SMase) (c) or (d), respectively, immobilizes or mobilizes the VDSs of Kv channels. It was modified from Refs [25••,26••]. SMase C produces ceramide and leads to the decrease of total gating charge (Q), indicating a decrease of active channels with mobile voltage sensor domains. SMase D treatment generates ceramide-1-phosphate, and liberates the VSDs in the channels as showed by the increased total gating charge. **(b)** KvAP voltage sensor domain switches from DOWN (resting) to UP (activated) conformation when it is changed from a nonphospholipid bilayer to a phospholipid membrane. The model at the bottom schematically demonstrates the lipid-dependent gating.

It was proposed that phospholipids directly interact with the arginines on the S4 [27••]. The requirement of phospholipids is therefore to stabilize the lipid-facing arginines in the 'UP' conformation, as implicated by electron microscopy and single particle reconstruction of detergent-solubilized KvAP, as well as in the EPR study of KvAP embedded in vesicles [37•,38•]. Multiple calculations by molecular dynamics simulation proposed that the phospholipids around the voltage sensor may interact with the hydrophobic cation in the guanidium side chains of the arginine residues on S4 that are partitioned to the middle of the membrane [39–41]. Such phosphate–arginine interaction could lead to a local distortion of the bilayer structure, decrease the energetic cost for the membrane insertion of arginines [42,43], and thus stabilize the 'UP' conformation in phospholipid membranes.

With nonphospholipid membranes, it is more difficult to stabilize the lipid-facing arginines through charge interactions since the phosphate groups on the lipids are absent. Instead, the interactions between S4 and nonphospholipids are dominated by hydrogen bonding and hydrophobic interactions. In this case the voltage sensors are likely in the 'DOWN' conformation where their S4 arginine residues experience less lipid-exposure. This observation is certainly oversimplified because the hydrophobic residues in the voltage sensors, especially those interposed between the arginines in S4, do contribute to the stability of the 'DOWN' conformations [7••,44]. Nevertheless, there are clear stabilizing interactions between the VSD and lipids in both the UP and DOWN conformations. It is thus imperative to study the structures of voltage-gated ion channels while they are embedded in a lipid bilayer.

Lipid molecules have been seen and described in a number of structures of membrane proteins. The lipids often co-purify with the membrane proteins of interest but sometimes the lipids are added to help stabilize the proteins for biochemical and structural analysis. The latter was the case with Kv1.2 and its chimera structure [28••]. The channel was difficult to handle as it often crashed out of solution making both biochemical and structural analysis difficult. However, addition of phospholipids during the purification stage markedly increased the stability of the channel making structural studies possible. In fact when the structure of Kv1.2 chimera was determined a number of lipid molecules were observed in the density maps. Lipids closely associated with both the VSDs as well as the pore domain of the channel (Figure 3a,b, lipids in yellow).

Figure 3



Lipids arranged around the Kv1.2 chimera structure. **(a)** The Kv1.2/2.1 chimera structure is viewed laterally from within the membrane. The protein density (PDB code 2R9R) is presented as a white surface map, and the partial (the majority) and full lipid molecules are showed as balls with yellow carbon atoms and red glycerol backbones. A putative lipid bilayer is marked gray. **(b)** The same model viewed from the extracellular side.

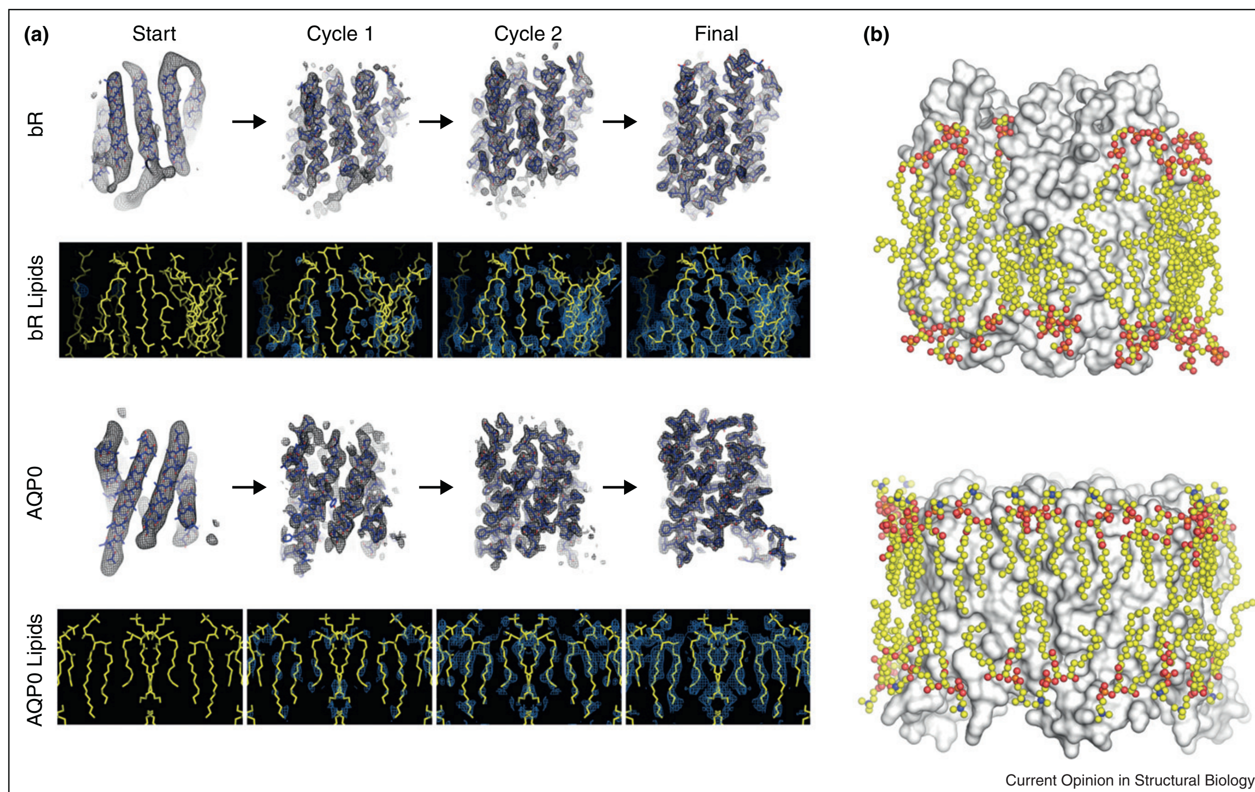
Electron crystallography of membrane proteins

Electron crystallography is the only structural biology technique in which the membrane protein of interest is crystallized within a complete lipid bilayer that mimics biological membranes [45,46]. Moreover, electron crystallography is the only electron cryomicroscopy (cryoEM) technique capable of delivering atomic information about membrane proteins. It has been used to provide important insights into the structure and function of several membrane proteins belonging to different protein families [47••]. Together with recent advance in hardware and methodology this approach shows a lot of promise. As discussed before, lipids are clearly important in the structural stability and function of voltage-gated ion channels. It is imperative therefore to begin to study the high-resolution three-dimensional structures of such channels by electron crystallography.

While many reviews have been written about the crystallization process for electron crystallographic studies this step remains challenging [48–52]. The most common way to achieve reconstitution and therefore crystallization of the membrane protein of interest is by slow dialysis. Here the detergent-solubilized purified membrane protein is mixed with detergent-solubilized lipids. The detergent is then slowly removed by dialysis against a crystallization buffer lacking the detergent. As the detergent is removed, lipids begin to form membranes and the protein of interest is integrated into this membrane. Under certain conditions (which have to be determined empirically) the protein molecules will pack tightly into two-dimensional (2D) arrays or crystals. Obviously the choices of detergents, lipids, lipid amount (measured in lipid-to-protein ratio), buffer composition and temperature all play a role in the 2D crystallization process. And 2D crystallization is no less complex than that for 3D crystals used in X-ray crystallography. Once large and well-ordered 2D crystals are obtained, data are collected under cryogenic conditions with low electron dose.

A recent study highlighted the potential of using electron diffraction for rapid structural analysis of membrane proteins [47••]. Here the authors relied on images of 2D crystals to supply initial low-resolution (but accurate) phase information. Electron diffraction to atomic resolution was then collected to provide accurate amplitude data. Polyalanine α-helical fragments were then placed into the low-resolution map and new phases were calculated and extended to the resolution limit of the electron diffraction data. The structures of three different membrane proteins were determined rapidly by this method following several cycles of phase combination, density modification, model building and refinement. When the resolution of the electron crystallographic study is sufficiently high (better than $\sim$3 Å), the structures of both protein and lipids can be determined [46] (Figure 4a). In both bacteriorhodopsin and the water channel aquaporin-0 (AQP0), complete lipid bilayers were observed surrounding the protein and detailed lipid–protein interactions were described (Figure 4b). A few key features were observed, for example, charge complementation

Figure 4



Phase extension in electron crystallography. **(a)** Phase extension for bacteriorhodopsin and aquaporin-0 (top and bottom, respectively). The method was recently developed for rapid structure determination by electron crystallography to atomic resolution [47••]. **(b)** Complete lipid bilayers were seen and modeled surrounding both bacteriorhodopsin and aquaporin-0 (top and bottom, respectively). Given sufficiently high resolution electron crystallography can reveal details on lipid–protein interactions and possibly how lipids influence the structure and function of membrane proteins.

between lipid and protein as well as hydrophobic matching principles.

Membrane proteins are dynamic as they undergo various conformational changes to carry out their biological function. This is beautifully illustrated by the work on VSDs and how they alter their conformations in the membrane to open or close the coupled ion-conducting pore. Perhaps one of the most important applications of electron crystallography in studying voltage-gated ion channels is in fact the presence of a complete lipid bilayer around the protein in the 2D crystals. Indeed a theme that is emerging in electron crystallography is that crystal contacts in membranes are mediated by lipids with very little or no direct protein–protein contacts [45]. Lipids in 2D crystals therefore buffer the protein from its neighbors and as such conformational changes of the protein in response to activators or inhibitors may not affect crystalline order. Structures of the protein of interest can then be determined under a variety of physiologically important functional states simply by incubating the crystalline membranes with activators or inhibitors, changing pH, or adding a variety of substrates. Functional as well as structural analysis of the protein of interest can therefore be carried out from the very same preparations. Moreover, electron crystallography can be used to visualize the atomic charged states of amino acids [45], a characteristic of this technique that has not been exploited yet but holds a great promise for the study of various voltage-gated ion channels to fully characterize their activation/deactivation and therefore function.

Concluding remarks

As the field of structural membrane biology expands and more and more structures of membrane proteins are determined our understanding of membrane biology grows. Yet at the same time our understanding is limited because the vast majority of structures are determined without the stabilizing effects of a surrounding lipid bilayer. Lipids and protein are an integral part of biological membranes: they are coevolved and function together to support life. Both the functions and structures of many membrane proteins are dependent on the presence of a lipid bilayer and it is imperative to study the structures of such proteins

within the context of real membranes by electron crystallography. This is especially true in the case of voltage-gated ion channels for a complete elucidation of the mechanism underlying their lipid-dependent gating.

Acknowledgements

Because of limited space we were not able to cite the large body of published work by many of our colleagues on the topics discussed here. The authors want to thank Dr. Liang Shi for his help in preparing the figures. The cryoEM work in Dr. Jiang's lab on the Kv channels is funded by NIH (R01GM088745 and R01GM093271). Dr. Gonen's group is supported by the Howard Hughes Medical Institute.

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