Minireview

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Crystallographic Analyses of Ion Channels: Lessons and Challenges*

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Membrane proteins fascinate at many levels, from their central functional roles in transport, energy transduction, and signal transduction processes to structural questions concerning how they fold and operate in the exotic environments of the membrane bilayer and the water-bilayer interface and to methodological issues associated with studying membrane proteins either *in situ* or extracted from the membrane. This interplay is beautifully exemplified by ion channels, a collection of integral membrane proteins that mediate the transmembrane passage of ions down their electrochemical potential gradient (for general reviews, see Refs. 1 and 2). Ion channels are key elements of signaling and sensing pathways, including nerve cell conduction, hormone response, and mechanosensation. The characteristic properties of ion channels reflect their conductance, ion selectivity, and gating. Ion channels are often specific for a particular type of ion (such as potassium or chloride) or a class of ions (such as anions) and are typically regulated by conformational switching of the protein structure between "open" and "closed" states. This conformational switching may be gated in response to changes in membrane potential, ligand binding, or application of mechanical forces. Detailed functional characterizations of channels and their gating mechanisms have been achieved, reflecting exquisite methodological advances such as patch clamp methods that can monitor the activities of individual channels (3). Until recently, corresponding information about the three-dimensional structures of channels was not available, reflecting difficulties in obtaining sufficient quantities of membrane proteins for crystallization trials. Happily, this situation has started to change with the structure determinations of the *Streptomyces lividans* K^+ channel (KcsA (4)) and the *Mycobacterium tuberculosis* mechanosensitive channel (MscL (5)).

A variety of reviews (6–12) have appeared recently that discuss functional implications of these channel structures. This review discusses these developments from a complementary perspective, by considering the implications of these structures from within the larger framework of membrane protein structure and function. Because of space restrictions, this review necessarily emphasizes membrane proteins that are composed primarily of α-helical bundles, such as KcsA and MscL, rather than β-barrel proteins, such as porins, typically found in bacterial outer membranes.

What Are KcsA and MscL? A Brief Introduction

KcsA and MscL are prokaryotic channels that fold as homo-oligomers (tetramers and pentamers, respectively) of relatively small subunits that contain two transmembrane-spanning helices. KcsA is a potassium-selective channel, consisting of a 160-amino acid subunit, that was identified in *S. lividans* by Schrempf *et al.* (13). KcsA shares the signature sequences with eukaryotic K^+ channels that are responsible for ion selectivity and pore formation. However, this prokaryotic channel lacks the regions of eukaryotic channels associated with voltage sensing and does not appear to be physiologically gated, although the open state is stabilized by low extracellular pH (14). MscL, the best characterized mechanosensitive channel, was isolated and characterized by Kung and coworkers (15, 16) from *Escherichia coli* membranes. This channel, composed of a 136-amino acid subunit, is gated by changes in lateral tension applied to the bilayer. When sufficient tension is applied to the membrane, MscL opens to form a large conductance, non-selective channel. MscL is thought to play a physiological role in protection against osmotic stress by functioning as a safety valve. Although many prokaryotic homologs of MscL have been identified, no obvious eukaryotic homologs have yet been found.

Admittedly, these proteins have not been at the forefront of channel research; for example, neither KcsA nor MscL is mentioned in Hille's classic work on channels (1). However, this situation has changed recently with the appreciation that prokaryotic channels offer many advantages for structural and functional studies. The explosion of channel sequences identified in prokaryotes and archaea through genome sequencing efforts represents fertile sources for future work, not only to help define the properties of more complex eukaryotic channels but also to characterize channels that are physiologically interesting in their own right.

Structural Analysis of KcsA and MscL

Before describing the KcsA and MscL structures, it is useful to discuss aspects of the crystal structural analyses to provide appropriate background for interpreting the structures. The basic steps in a protein crystal structure determination are to (*a*) prepare protein; (*b*) grow crystals; and (*c*) solve the structure. The intrinsic properties of membrane proteins pose unique challenges at each step for the structure determination of ion channels and other integral membrane proteins, as summarized in the following paragraphs.

Protein Preparation—Perhaps the single greatest challenge for the structure determination of ion channels is the difficulty in obtaining sufficient quantities of material. This situation fundamentally reflects the limitations of current systems for the overexpression of membrane proteins of relatively low abundance (17). As a consequence, most membrane proteins of known structure are naturally present in relatively high abundance so that overexpression methods are not essential. Recently, exceptions to this generalization have been provided by the KcsA and MscL channels that have been successfully overexpressed in *E. coli*. In both cases, the channels were solubilized directly from the membrane fraction using alkyl maltosides. In this context, it should be noted that several β-barrel proteins from bacterial outer membranes have been successfully overexpressed and refolded from inclusion bodies (18). Purifications of KcsA and MscL were greatly facilitated by the addition of polyhistidine tags, followed by metal affinity chromatography. As with most crystallization-associated endeavors, the choice of detergents and the type and location of the affinity tags are variables that need to be experimentally explored. Successful crystallization studies are heavily dependent upon the availability of homogeneous, active protein preparations. Mass spectrometry provides a powerful approach for characterizing the integrity and purity of the protein preparation (19). Unfortunately, it is not possible to check activity by directly measuring the ionic conductance of channels solubilized in detergents, but the binding of toxins or other inhibitors (when available) can provide a good measure of functional competence.

Crystallization—Because there are no magic bullets for crystallizing ion channels, we have adopted the general approach of screening a diverse set of protein samples for crystallization under a more limited set of conditions rather than exhaustively screening a single sample, *i.e.* the underlying philosophy is that if the protein "wants" to crystallize, this can be established relatively quickly. The basic approaches to varying the protein sample include adding something, removing something, or trying something different (but related). Additives are often employed in crystallization trials (20);

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for the case of MscL, the heavy atom reagent gold sodium thiosulfate led to a substantial increase in diffraction quality, likely reflecting the stabilization of lattice contacts by binding at the interface between two pentamers. The use of D_2O in place of H_2O in the crystallization solutions (21) also improved crystal quality, perhaps reflecting the enhanced thermal stability of proteins in heavy water (22). Other "additive" approaches not yet described for ion channels would include the crystallization of channel inhibitor-toxin complexes or the formation of channel Fv-antibody complexes, as described for the crystallization of the *Paracoccus denitrificans* cytochrome *c* oxidase (23). An excellent example of the utility of "removing something" is provided by the KcsA structure; successful production of suitable KcsA crystals required protease treatment to produce a proteolytic fragment primarily containing the transmembrane domain. The use of protein homologs also provides a straightforward way of varying the protein sequence in crystallization trials. This approach was used by Kendrew and co-workers (see Ref. 24) in the very first protein structure determination of myoglobin and was also employed in the MscL analysis, where the *M. tuberculosis* protein was one of nine homologs that were cloned, expressed, purified, and screened for crystal formation.

Structure Determination—The crystallographic approaches used to solve structures of ion channels and other integral membrane proteins are no different from those employed for water-soluble proteins and other macromolecules. The most significant impact on the quality of the final structure is imposed by the moderate diffraction quality (3–3.5-Å resolution) and associated high overall temperature factors (~100 $Å^2$) observed for the KcsA and MscL structures. The moderate diffraction quality may reflect the relatively few packing contacts and high solvent content (~75–85%) observed for many, but by no means all, membrane proteins. In addition, multiple conformational states or orientations of the protein may exist in a crystal. The transmembrane regions of both KcsA and MscL are surrounded by rather extensive regions of partially ordered density, which likely represent detergent, tightly bound phospholipids, or even disordered polypeptide from the termini of the proteins. As a consequence of these effects, the final R factors after refinement for channel structures tend to be high, as are the associated uncertainties in coordinate positions. In the case of the MscL structure determination, it was not possible to get R factors below 0.4 by refining single models, and instead multiple models with tight non-crystallographic symmetry were utilized. It seems likely that as more complex and less well ordered macromolecular assemblages are studied structurally, appropriate methods for modeling distributions of structures, including partially ordered models, will need to be developed.

Structural Organization of Channels

General Features of Membrane Protein Structures

To fold within the ~30-Å wide hydrophobic environment of the membrane bilayer, integral membrane proteins such as KcsA and MscL are constructed from a framework of membrane-spanning α-helices that average ~25 residues in length and contain predominantly hydrophobic amino acids (see Refs. 25 and 26). The helical axes are not randomly oriented with respect to the membrane bilayer but tend to be aligned along the normal to the membrane plane, with an average tilt angle of ~21° (27). Because of the orientational requirements imposed on membrane-spanning helices, helix-helix packing angles seen in membrane proteins (27) are more restricted than observed for water-soluble proteins. Reflecting the overall apolarity of the transmembrane region, the interiors of membrane-spanning proteins (neglecting polar residues lining channel pores) are apolar and have an average hydrophobicity comparable with that observed within the interior of water-soluble proteins (28). Relatively few polar interactions (hydrogen bonds, salt bridges) are found between adjacent α-helices. The surface (lipid-facing) residues of membrane-spanning proteins are found to be somewhat more apolar than the interior residues. The similarities between water-soluble and membrane proteins in terms of interior hydrophobicity and packing density (29) suggest that water-soluble proteins can be considered as modified membrane proteins with covalently attached polar groups that confer solubility in aqueous solutions (30). A direct consequence of these similarities would be that it should be possible to convert membrane proteins into water-soluble proteins by replacement of lipid-facing residues with polar residues through mutagenesis; promising attempts toward this goal have been recently reported (31). If successful, this suggests an alternate strategy for the crystallization of ion channels and other integral membrane proteins, which would be to create water-soluble versions through mutagenesis.

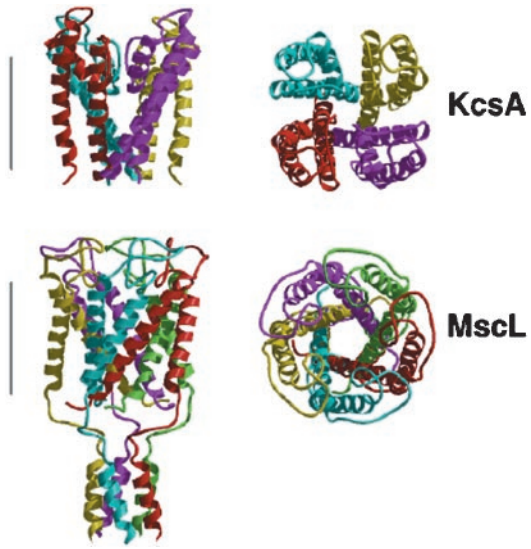

FIG. 1. **The KcsA tetramer (*top*) and MscL pentamer (*bottom*), viewed from directions perpendicular (*left*) and parallel (*right*) to the normal of the membrane plane, respectively.** The *vertical bars* on the *left* are 30 Å in length and mark the approximate membrane-spanning region of these channels. Figs. 1, 2, and 3 were prepared with MOLSCRIPT (52) and RASTER3D (53).

Structures of KcsA and MscL Channels

With two transmembrane helices per subunit, KcsA and MscL have practically the simplest membrane- spanning topology possible for a channel, with the exception of channels such as the influenza virus M2 protein (32) that contain a single transmembrane helix per subunit. In both KcsA and MscL, one transmembrane helix (the "inner" helix) lines the permeation pathway, whereas the second helix (the "outer" helix) is positioned on the outside of the channel, and the polypeptide termini are cytoplasmic in both cases. Outside of these similarities, there are distinctive features in the polypeptide folds of these two proteins.

KcsA—In the structure of this tetrameric protein (Fig. 1, *top*), the first (outer) transmembrane helix leaves the cytoplasm and crosses the membrane to form the outside of the channel. The second (inner) transmembrane helix returns to the cytoplasm by crossing the membrane to form the permeation pathway. The polypeptide region between the transmembrane helices is highly conserved among K^+ channels and creates the selectivity filter and pore helix that are crucial to the specificity of this channel for potassium. The dominant interaction stabilizing the protein structure in the transmembrane region occurs between adjacent inner helices; although the outer helices interact extensively with the inner helix of the same subunit, there are no intersubunit contacts between adjacent outer helices.

MscL—The structure of the MscL pentamer (Fig. 1, *bottom*) consists of two domains, transmembrane and cytoplasmic, that share the same 5-fold axis relating subunits within the channel. The first transmembrane helix of each MscL subunit starts in the cytoplasm and crosses the membrane as the "inner" helix to form the permeation pathway of this channel, whereas the second helix returns to the cytoplasm across the membrane to form the "outer" helix. Hence, MscL is threaded across the membrane in the opposite manner to KcsA. The extracellular loop connecting the transmembrane helices exhibits extensive sequence variability within the MscL family, and the functional significance of this loop is unclear. Unlike KcsA, the outer helices do contact the inner helix of an adjacent subunit. The cytoplasmic domain consists of a five-helix bundle that extends for ~35 Å away from the likely plane of the membrane-aqueous interface.

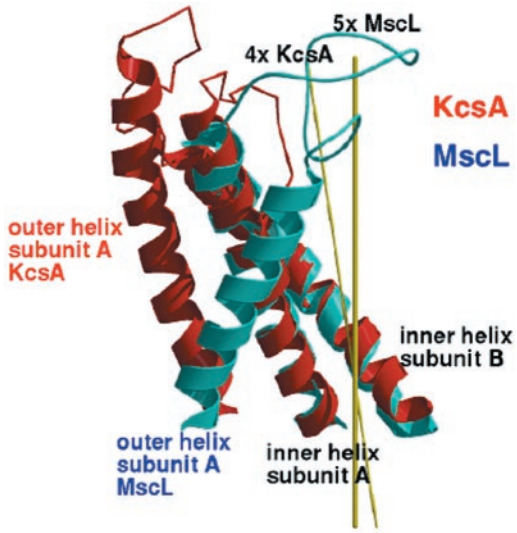

FIG. 2. **Superposition of inner helices from adjacent subunits, labeled *A* and *B*, for the KcsA (*red*) and MscL (*cyan*) channels, respectively.** Similarities are evident in the packing arrangements between these inner helices, despite the opposite threadings of the polypeptide chains in the two channels. The outer helices from the A subunit are included to highlight the different relative positions of these elements between KcsA and MscL. The positions of the 5-fold axis of MscL and the 4-fold axis of KcsA are indicated by the (nearly) vertical *yellow* lines.

Helix Packing in Channels

In many channels, the permeation pathway for ion conductance is generated from α-helices packed together around a central axis. Often, these helices are formed from equivalent residues on identical (or at least homologous) subunits that are related by an n-fold rotation axis. If the helices are tilted by an angle η with respect to the membrane normal and if the subunits are related by a rotation of $\theta = 360/n°$ about the normal, then the crossing angle, α, between adjacent helices can be determined from the relationship: $\cos\alpha = \cos^2\eta + \sin^2\eta \cos\theta$ (33). Because $\eta = \sim 35°$ for the inner helices of KcsA and MscL, then α is calculated to be $\sim\pm 40°$ for these cases. The negative and positive angles correspond to right- and left-handed helical bundles, respectively, with the right-handed bundles actually observed for both KcsA and MscL. Although crossing angles with $\alpha = \sim -40°$ are infrequently observed in membrane proteins (27), this does correspond to a favorable ridges-in-grooves type helix packing arrangement (34). Indeed, the inner helices observed in channels structurally characterized to date (KcsA and MscL, along with lower resolution electron microscopy studies of the acetylcholine receptor (35) and aquaporin (36)) do pack together as right-handed helical bundles. One consequence of the relatively steep packing angle exhibited by the pore-forming helices is that the contact interface between helices is localized to a fairly narrow region, which may facilitate helix-helix rearrangements associated with channel gating. As with other membrane proteins, residues buried at the interface between interacting transmembrane-spanning helices are predominantly apolar, with relatively few hydrogen bonds or other polar types of contacts observed. The apolar and non-directional nature of these helix-helix contacts may also facilitate helix rearrangements associated with channel gating.

Despite the differences between KcsA and MscL in oligomeric state and threading of the transmembrane helices, the basic nature of the contacts between helices lining the permeation pathway is quite similar between the two proteins, as reflected in the helix-helix crossing angles of $\sim -40°$. The similarities in helix packing can be illustrated by a superposition of adjacent inner helices in the two channels (Fig. 2). The relative positions of the outer helices with respect to the inner helices differ significantly between KcsA and MscL, however. Although the outer helix in KcsA is positioned such that it only contacts the inner helix of the same subunit, the outer helix of MscL is rotated by an additional $\sim 90°$ around the intrasubunit inner helix, relative to KcsA. As a consequence, the outer helix of MscL contacts the inner helices of two neighboring subunits. A similar role for the outer helix in mediating subunit-subunit interactions has been proposed for the outer helix in the Kir family of inward-rectifying channels (37).

Functional Implications of Channel Structures

Ion Selectivity—The crystal structure of KcsA has revealed features of the molecular architecture of a potassium channel that are responsible for ion selectivity and permeation. Near the extracellular opening of the channel, a "selectivity filter" composed of the peptide carbonyl oxygens of the K^+ channel signature sequence is held in the appropriate position to preferentially coordinate dehydrated K^+ ions relative to either smaller (Na^+) or larger ions. Below the selectivity filter, the movement of positive ions across the apolar membrane is stabilized by a polar, water-filled cavity of $\sim$10-Å diameter and the electrostatic effects of the appropriately oriented carbonyl oxygens of the pore helix. Consequently, ion selectivity and conductance are achieved through the favorable coordination geometry of potassium by the selectivity filter and lowering the dielectric barrier for the passage of ions by electrostatic optimization of the channel. A computational analysis quantitating the contributions of these effects has recently appeared (38).

Gating—An important motivation for initiating the structural analysis of MscL was to understand channel gating, because MscL opens and closes in response to mechanical stresses applied directly to the membrane. The high conductance and lack of ion selectivity are consistent with a large, water-filled pore existing in the open state of the MscL channel (16). Because the open state has a high conductance corresponding to a pore diameter of up to 40 Å (39, 40), it is anticipated that there will be substantial conformational changes associated with the transition between closed and open states. Consequently, MscL should be an excellent system for analysis of gating transitions, because biochemical studies complemented with structural studies at even moderate resolutions could reveal basic features of this process. An important development in establishing the gating mechanism of MscL has been the identification of "gain-of-function" mutants that display a slow or no-growth phenotype in a liquid medium that is likely due to the leakage of solutes out of the cell (41). *In vitro* characterization of these mutant channels demonstrated that they generally exhibit a reduction in the tension required for channel gating, suggesting that the closed state in these mutants is destabilized relative to the wild-type channel. Many of the mutations associated with severe phenotypes are located at the interface between adjacent inner helices in the region of the membrane-spanning domain where the pore is most restricted. Javadpour *et al.* (42) have noted that glycine residues are frequently found at the interface between transmembrane helices. Intriguingly, an extensive mutagenesis study of residue Gly-22 in *E. coli* MscL (corresponding to Ala-20 in *M. tuberculosis* MscL, which is at the interface between adjacent inner helices) revealed that hydrophobic and hydrophilic substitutions stabilized the closed and open states of the channel, respectively, suggesting that this residue becomes exposed in the open state (43). These observations suggest that contacts between inner helices play a crucial role in the gating mechanism. This interface must be rearranged, perhaps by allowing the inner and outer helices to interleave, to create a pore of sufficiently large diameter in the open state (see Ref. 40). Movements of the inner helices between closed and open states have been reported for the acetylcholine receptor (35) and for the KcsA (44) and *Shaker* K^+ channels (45).

A key aspect of the function of MscL is the coupling mechanism between protein conformation and membrane stretching, which must be mediated by changes in the interactions between the channel and the membrane. Although not sufficiently well ordered to model crystallographically, there is a significant amount of diffuse electron density located on the cytoplasmic side of the transmembrane domain, which could represent partially ordered lipid or detergent. Rearrangements in the lipids packed around the channel in response to stretching the membrane could provide a mechanism for coupling protein and membrane structures. An applied tension of $\sim$12 dynes/cm is required to open the channel (46), which approaches the tension needed to rupture the membrane. This suggests as a working model that the lateral pressure in the membrane bilayer (47, 48) clamps the channel in the closed state; when

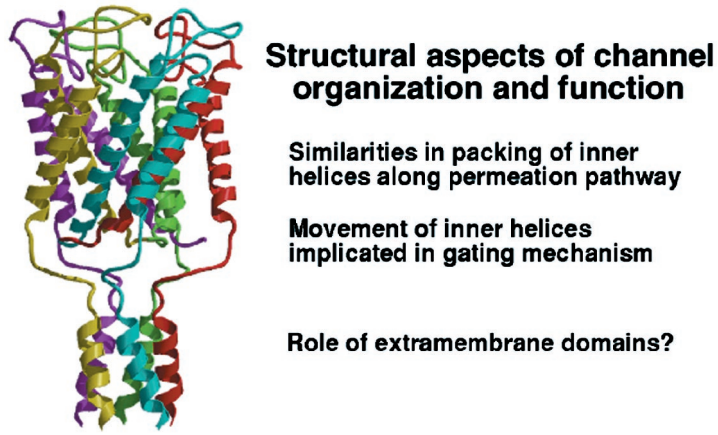

FIG. 3. **Similarities in the structural organization of ion channels characterized to date, mapped onto the MscL structure.** Common features of channels discussed in the text include the packing interactions of inner helices to form the permeation pathway, the likely movement of the inner helices as part of the gating mechanism, and the presence of extramembrane domains adjacent to the permeation pathway through the membrane.

the membrane is stretched, this pressure is reduced, allowing the channel to expand to the open state.

Extramembrane Domains—Although substantial emphasis has been placed, with considerable justification, on the role of the transmembrane region in ion conduction, extramembrane structures also influence the conductance and gating properties of channels. In MscL, the cytoplasmic domain consists of a five-helix bundle juxtaposed against the membrane, such that the pore extends continuously through both domains. The functional significance of this domain is unclear, because deletion studies suggest that much of this domain can be removed from the *E. coli* homolog without substantially altering activity. It is striking, however, that two structures that interact with voltage-gated K^+ channels from the cytoplasmic surface, the tetramerization domain of the *Shaker* K^+ channel (49) and the β subunit (50), exhibit the same 4-fold symmetry as the membrane domain and have continuous channels along the rotation axis. In addition, recent analyses by electron microscopy of the acetylcholine receptor (51) demonstrate that the cytoplasmic portion of the channel contains fenestrations that could serve as part of the permeation pathway. Although the functional significances of these observations are uncertain, they do raise intriguing questions about the structural organization of the extramembrane regions and how these may influence channel conduction and gating.

Future Challenges

This is unquestionably an exciting time to be working on ion channel structures, as it is now possible to begin interpreting the wealth of functional measurements in terms of specific molecular models (Fig. 3). Beyond the continued characterization of prokaryotic channels, the analysis of voltage and ligand-gated channels provides particularly attractive targets for structural studies. An important future objective is the structure determination at high resolution of a gated channel in both closed and open states. The availability of both structural and electrophysiological data will allow realistic computational studies relating structure and function that should provide the ultimate test of our understanding of how ion channels work.

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