CHEMICAL REVIEWS

Review

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Analytical Approaches for Studying Transporters, Channels and Porins

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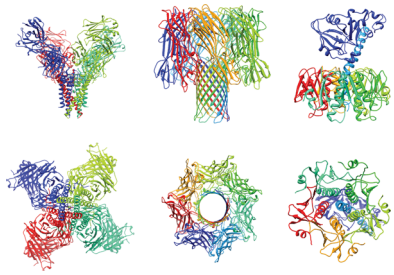

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1. INTRODUCTION

Membrane-bound proteins are the basis of signaling, bioenergetics, and transport of ions and reaction products across cell walls.[1,2] They accomplish these critical tasks by pumping ions[3] and small molecules against their respective concentration gradients; selecting, with high specificity, species to be transported across cell membranes (e.g., ions,[4] sugars,[5−7] and water[8]); gating (i.e., opening and closing in response to signaling molecules such as neurotransmitters[9]) to offer further control over ion transport;[4] and disrupting cellular processes due to the formation of large holes in membranes (e.g., hemolysins).[10] Although porins are functionally and structurally different than ion-selective and gating ionic channels found in excitable membranes or nerve and muscle,[1,2] they are also considered in this review.

Because ion-conducting proteins are ubiquitous in the body, their dysfunction causes a wide range of diseases including neurological (e.g., Alzheimer's disease),[11,12] cardiac,[13−20] and others (e.g., cystic fibrosis[21,22]). However, fully functional channels also play a role in infectious diseases with viral channels aiding the transmission of HIV, in bacterial infections (e.g., *Vibrio cholerae* cholera toxin,[23] *Escherichia coli* heat-labile enterotoxin,[24] *Bordetalla pertussis* pertussis toxin,[25] *Corynebacterium diphtheriae* diphtheria toxin,[26] *Staphylococcus aureus* α-toxin (α-hemolysin) from,[27−29] part of the *Bacillus anthracis* anthrax toxin complex[30]), and in plant-derived toxins such as ricin.[31,32] Research into the nature of channel-induced diseases (channelopathies) now has at its disposal a wide range of tools to study the structure and function of these proteins and the diseases associated with them.[33−42]

To combat diseases caused by channels, it is important to develop an understanding of how they work on the molecular level. Electrophysiology, the principle tool to study channel function,[1,43] was developed to monitor the ionic current that flows through the channels and to characterize the channel responses to agonists. In effect, it is a direct measure of channel function. However, measuring the result of the channel's function does not provide sufficient information about *how* the protein actually works. For example, since the early 1950s, electrophysiological measurements demonstrated conclusively that there were transport conduits (ion channels) that were highly specific for different monovalent cations (e.g., Na^+ or K^+). However, the mechanisms for this remarkable discrimination capability were elusive until the late 1990s, when MacKinnon and colleagues published a high-resolution structure of a potassium channel structure.[44] This work, which led that team to describe the basis of potassium selectivity, was awarded the Nobel Prize in 2003. Because such

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high selectivity requires precise 3-dimensional alignment of multiple segments of these proteins, it is apparent that determining high-resolution structures is a critical tool for understanding the function and dysfunction of channels. These classical methods are now complemented by many more disciplines, as diverse as functional genetics and spectroscopic techniques, which improve our understanding of how channels work on the molecular level,[45] how they are implicated in disease,[46−48] and how to harness the structural information for rational drug design.[49,50]

Membrane protein structural biology is a frontier area of modern biomedical research; 20−35% of the proteins encoded by an organism's genome are integral membrane proteins.[51,52] These macromolecules (channels, transporters, and receptors) are critical components of many fundamental biological processes, as described previously. As the majority of drug targets are integral membrane proteins, the latter are critically important in biomedical and biotechnological applications.[49]

To function properly (i.e., to discriminate between subtly different ions and change conformation in response to binding a neurotransmitter or a change in transmembrane potential), ion channels have an impressive array of folds, binding sites, selectivity filters, and other structural nuances. The ability to discover new ion channels is progressing at breakneck speeds in the post-genomic era, driven by the ability to find membrane proteins directly from the gene sequence,[52−54] and the resulting structural space can be used to predict function.[55−58]

Ultimately, membrane-spanning proteins have two major structural motifs, β-barrels/β-sheets or α-helices (figure 1).

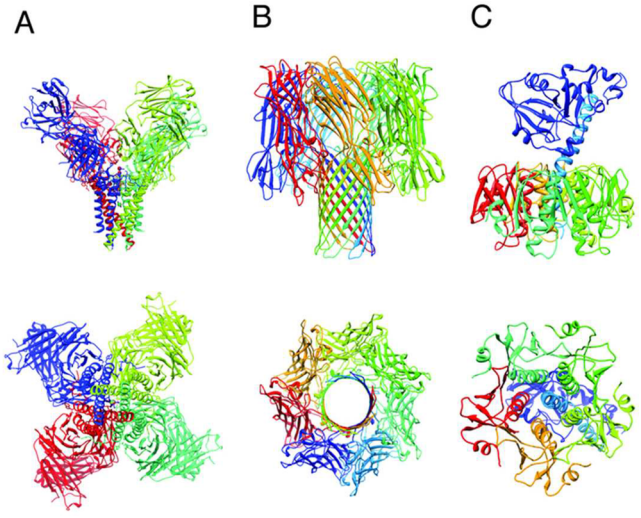

Figure 1. Membrane-transport proteins display a diverse range of structures to shepherd charged and polar molecules across membranes. Typical channel structures are based on (A) α-helixes as with the K^+ channel KcsA,[169,170] (B) β-barrels as with *Staphylococcus aureus* α-hemolysin (αHL)[28] or (C) combinations of both as with the cholera toxin.[23]

From these structures—and associated measurements—the various functions of the proteins can be elucidated, and as the molecular mechanism for the disease state is understood, therapeutic schemes can be developed to treat channelopathies. Several excellent reviews discuss families of ion channels:[4] K^+ channels,[59−62] glutamate receptors,[63] Na^+ channels,[64−66] and Cl^- channels.[67,68] As such, this review will focus on the development of measurement modalities for membrane protein structure determination, which relates directly to deducing structures of ion channels.

The field of structural biology has been largely driven by the development of powerful analytical technologies that can be classified by several criteria (Figure 2), and it is important to

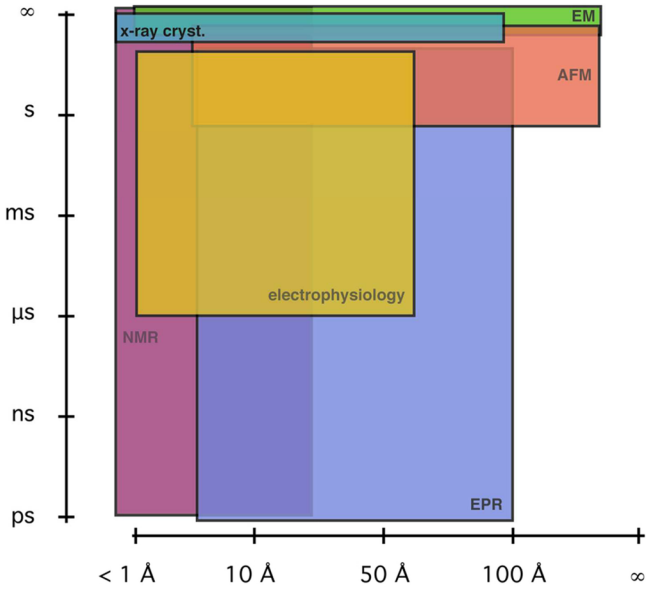

Figure 2. Wide array of techniques that can be used to analyze membrane proteins cover a range of length and time scales. Many of these methods are complementary[328] and offer distinctly different information about the structure and function of membrane proteins.

have a clear understanding of how, why, and when each measurement is done and which method(s) or suite of methods is best used to solve a given problem.[69] These technologies span the electromagnetic spectrum from X-rays,[70] to visible and infrared radiation,[71−73] and radiowaves;[74−76] use larger particles subatomic particles such as electrons[77] and neutrons;[78] use mechanical apparatuses;[79] and of course include the measurement of ionic currents.[43,80] Each of these techniques provides both unique and complementary information that generates critical knowledge about the proteome, can be used to develop a more complete understanding of disease on the molecular level, and aids in the development of new treatments against both genetic and infectious diseases. This review will briefly touch upon each of these classical approaches and present emerging techniques for the study of membrane protein and channel structure with particular emphasis on determining the *functional* form.

Regardless of the analytical techniques used to probe ion channels for structure and function, sample preparation is the principle bottleneck in the study of these proteins. With the obvious exception of on-cell patch-clamp techniques,[43,80] membrane protein analytical techniques require protein expression, purification, and preparation. The issues in expressing significant quantities of membrane proteins are not completely understood.[81] Coaxing a cell to produce a non-native protein in large amounts is nontrivial. Although challenging for soluble proteins, the task can be nearly insurmountable for membrane protein systems. Our knowledge of the necessary cofactors, enzymes, and lipid structures is still not complete enough to design a fully rational recombinant system. In vitro transcription and translation remains an

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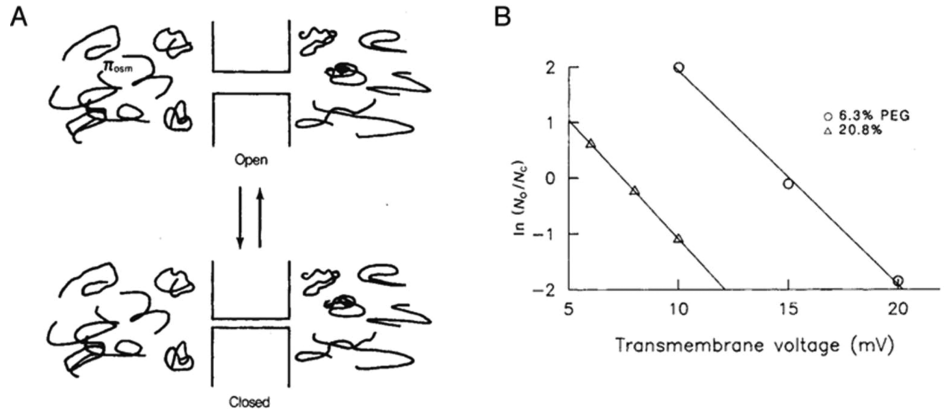

Figure 3. Determining the change in ion channel pore volume using osmotic stress. (A) The osmotic pressure gradient caused by polymers that are too large to enter the pore cause the exodus of water molecules from the pore to the bulk. The movement of the channel accompanies the loss of water from the open to the less open state, as well as a change in volume equal to that of the lost water. (B) Increasing the applied potential shifts the equilibrium between the open and closed states of the channel to favor the latter. Increasing the external osmotic pressure shifts the equilibrium, such that the channel is easier to gate with the applied potential. The shift in the electrical energy required to gate the pore is caused by the pressure-induced volume change of the channel pore. Reprinted by permission from ref 91. Copyright 1986 Macmillan Publishers Ltd.: Nature.

appealing technology, but to date it does not yet produce enough protein for most structural techniques.[82] Unsurprisingly, the best route to success is to use a host that closely resembles the organism from which the protein under study originates.[83] Even if a protein can be expressed in abundance, for it to provide any scientific value it must also fold into its native or functional form and maintain that fold in the presence of solubilizing detergent, or lipidic matrix use for the purification and analysis. The more complex the protein, the more difficult that prospect has been.

2. FUNCTIONAL PROTEINS: ELECTROPHYSIOLOGY

When investigating a new or hypothetical ion channel, the first and most productive line of research is to establish the true function of the protein. Ion channel electrophysiology began with the revolutionary work by Cole, Hodgkin, Huxley, and others.[84−86] These seminal studies demonstrated that there were separate pathways for sodium and potassium ions to transport across membranes, that these pathways can "gate" or switch between open and closed states, and that the coordination of these processes was the molecular basis of nerve impulse conduction.

In lieu of a high-resolution map of a protein ion channel's structure, some of the more salient features (e.g., limiting aperture of the channel pore, pore topology, ion selectivity, and distribution of charges along the pore) can be determined using electrophysiology, site-directed mutagenesis, electrochemical impedance spectroscopy, and molecular-scale probes of various types. In addition, even if a channel's structure has been deduced using X-ray crystallography or other techniques, the other methods can be used to critically test the putative structures and possibly determine whether the molecules used in the structure studies were indeed functional.

2.1. Estimating Channel Size: Geometrical Considerations

In the simplest case, the pore radius (r) can be estimated from the measured single-channel ionic conductance (g) and by assuming that the channel length (L) spans the membrane (~4 nm thick), that the pore is a smooth right circular cylinder with cross-sectional area $A = \pi r^2$, that the conductivity of the solution in the pore (σ) is identical to that in the bulk, that all of the applied potential drops across the pore, and that Ohm's law applies, such that $g = A\sigma/L$ or $r = (gL/\sigma\pi)^{1/2}$. More precise methods for estimating the channel pore geometry are described below. Although helpful, this first-order estimate of pore size is often overly simplistic.

2.2. Pore Size Estimated by Osmotic Stress-Induced Gating

As part of their normal function, many channels "gate" (i.e., switch) between different conductance states. Is this change in conductance caused by a small or large change in the channel's structure (i.e., like a sluice gate controlling the flow of water in a trough, or a wholesale change in the entire pore diameter and/or length)? Or could it be due to both?

By measuring transient changes in the ionic current caused by a step change in the transmembrane applied potential, Bezanilla and co-workers demonstrated that, in some cases, voltage-dependent channel gating can be caused by the movement of a small number of charges in the transmembrane electric field.[87−89] On the other hand, Zimmerberg and Parsegian showed that the volume change that accompanies the voltage-induced gating of the voltage-dependent ion channel from mitochondria (VDAC),[90] which is estimated by applying an osmotic stress with nonelectrolyte polymers that are too large to enter the pore,[91,92] can be substantial (Figure 3).

Assuming that a given channel can switch reversibly between an open and closed state, then the number of channels in those two states (N_o and N_c, respectively, or the proportion of time a single channel spends in the states) follows a Boltzmann distribution,

$$N_o/N_c = \exp(-nq(\psi - \psi_0)/k_BT)$$

where n is the number of "gating charges" with charge q,[88] ψ is the applied potential, ψ_0 is the potential at which half the channels are open, k_B is the Boltzmann constant, and T is the absolute temperature. At a given applied potential, pore-impermeant polymers provide an additional stress on the channel that results in the removal of water from the channel. Because nature abhors a vacuum, this process favors the closed state, such that

$$N_o/N_c = \exp[(-nq(\psi - \psi_0) - \Pi_{osm}\Delta V)/k_BT]$$

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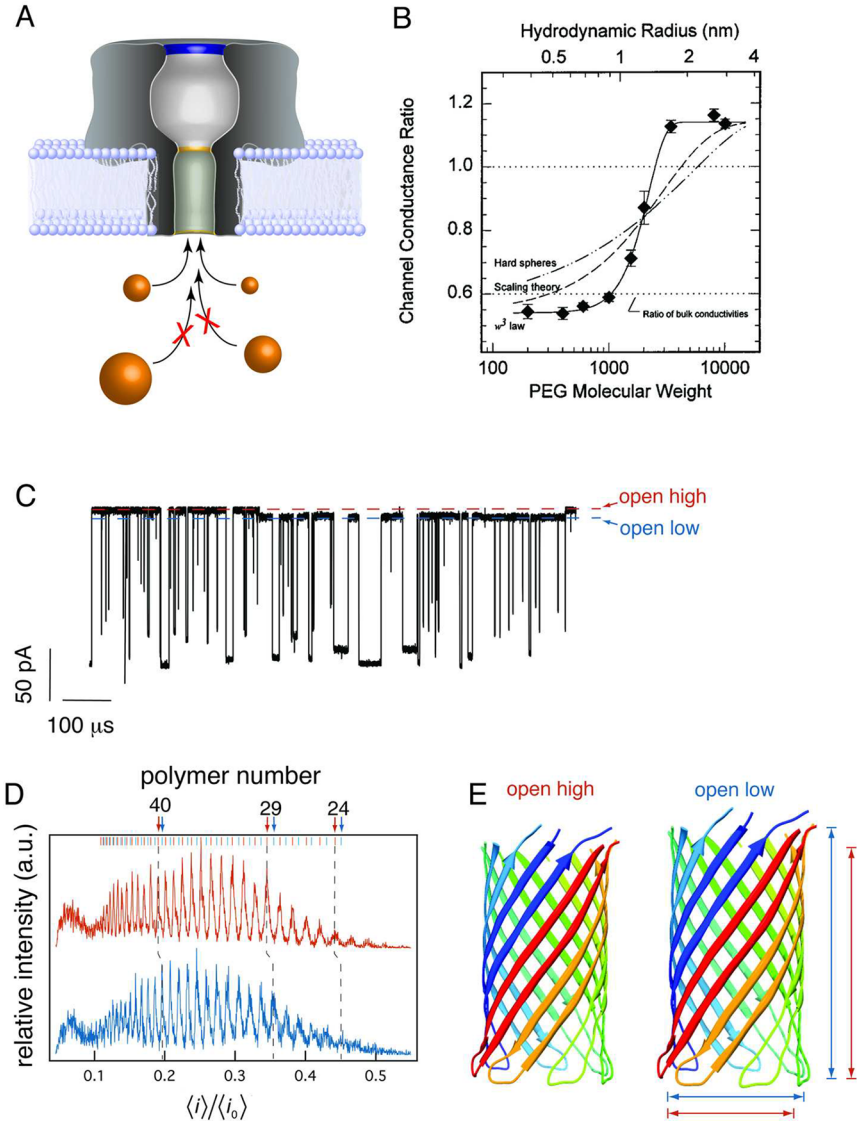

Figure 4. Determining the limiting aperture in an ion channel based on the ability of different size polymers to partition into the pore (A). This method was pioneered by Krasilnikov and colleagues.[101] When the polymer–pore interactions are short, noise analysis can be used to measure a partitioning curve and produce a direct estimate of the pore size (B).[100] Increasing the binding constant causes long-lived resistive pulses that originate from separate open states of the ion channel (C), which can be characterized both in terms of open channel conductance and polymer-induced blockade depths. The blockade depths show a subtle shift in their position between the two open states (D), which can be tied to structural changes in the pore. In this case an elongation and slight constriction of the β-barrel of αHL, the illustration exaggerates the actual conformational shift of the pore for clarity (E).[105−107] Reprinted with permission from ref 107. Copyright 2010 IOP Publishing.[7]

where Π_{osm} is the osmotic pressure difference between pore interior and the bulk and ΔV is the difference in channel pore volumes of the open and closed states. Thus, the polymers perform real pressure–volume ($\Pi_{osm}\Delta V$) work on the pore. For VDAC, channel gating occurs with the loss of $\sim 10^4$ water molecules from the channel pore.[91] Similar methods have been used to estimate the change in pore volume of other ion channels.[93−96] In addition, it was shown that gating can be caused by the translocation of a segment of a pore-forming toxin across the membrane.[97] Thus, when channels gate, either small, medium, or large amounts of protein can move.

2.3. Pore Radius Estimated by Ions and Polymers

In the case of porins that have a sufficiently large lumen such that water remains bulk-like (e.g., pore-forming toxins), the limiting aperture has been determined using ions[98] and pore-permeant water-soluble polymers[99−103] with different hydrodynamic radii. Ions that are small enough to enter the pore, but large enough to become lodged in the pore's limiting aperture, reduce the channel conductance. The polymer-based method developed by Krasilnikov and colleagues[99,102] is conceptually different. Specifically, non-electrolyte polymers of well-defined size, and that change the bulk conductivity, are added to the solutions bathing the channel. If the polymers are too large to enter the pore, the channel conductance will remain essentially unchanged. If the polymer is smaller than the limiting aperture, then it will enter the pore and reduce the channel conductance, more or less by the same amount it reduces the bulk conductivity (Figure 4B). The largest polymer that can enter the pore provides a direct measure of the channel's limiting aperture, assuming the "size" of the polymer is known. Poly(ethylene glycol) has been used extensively in ion channel pore-sizing experiments because it is readily available in a wide

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range of molecular masses and its hydrodynamic radii are known.[104] By modifying the solution conditions such that the polymer probe spends a long time in the pore (i.e., μs to ms), the nanopore can operate like a single-molecule mass spectrometer (SMMS).[105] Resistive pulses caused by single polymers diffusing into the pore can be characterized according to the relative conductance ratio of the blocked channel to the open channel (Figure 4C). In the case of poly(ethylene glycol) (PEG) partitioning into *Staphylococcus aureus* α-hemolysin porin, the blockade depth is controlled by both volume exclusion (size) and the interaction of the polymer with electrolyte (i.e., the polymer acts like a fixed buffer within the pore, further reducing the current).[106] Under the proper conditions, PEG can be resolved over a wide range of polymer numbers from <10 to >70. When a channel shows gating behavior or has multiple open states (Figure 4C), the SMMS blockades shift based on the change in volume of the pore (Figure 4D).[107] Although these changes do not appear significant at first glance, the open channel states and polymer-induced blockade depths can be used to probe fine structural changes within an ion channel's water-filled lumen. For the channel formed by α-hemolysin, this SMMS approach can detect changes in the pore geometry of ~2 Å in length and −1 Å^2 in cross-sectional area, suggesting that the stable open channel can stretch and narrow much like a Chinese finger trap (figure 4E).

2.4. Pore Topology

Of the general structural motifs that proteins adopt, the membrane-spanning pore segment of ion channels and porins are either α-helix or β-sheet (figure 1). When possible, the structural topology is deduced from X-ray crystallography.[28,44] However, as discussed elsewhere in this review, obtaining crystal structures of membrane proteins, including ion channels, has proven to be most difficult.

The nature of hydrogen bonding in α-helices or β-sheets causes the amino acid side-chains in them to repeat their orientation every 3.7 or 2 residues, respectively. Akabas, Karlin, and co-workers took advantage of this and realized that the pore topology of an acetylcholine receptor channel could be determined using a combination of site-directed mutagenesis and subsequent chemical modification.[108] Specifically, they demonstrated that water-soluble, charged sulfhydryl-specific reagents, each about 0.6 nm in diameter and 1 nm long, reacted with single novel cysteines added to the primary sequence (and thereby block the channel conductance) if and only if the cysteines were accessible to the bulk aqueous phase instead of the lipid membrane. The results showed that these reagents reacted with every other amino acid side-chain over a section of the primary sequence, which suggested that the M2 segment of the channels was likely a β-sheet—in part because the only crystal structures of channels were β-barrel porins.[109,110] Later electron-diffraction studies determined that the channels were in fact α-helical, highlighting the need for multiple lines of research.[111,112] Similar studies showed that the membrane-spanning segment of the channels formed by *Staphylococcus aureus* α-hemolysin[113] and *Bacillus anthracis* protective antigen 63 are β-barrels, and these hypotheses here hold.[114]

Additional structural details of the channel pore can be deduced using simple polymer constructs. Kasianowicz and colleagues demonstrated that single-stranded RNA and DNA can be driven electrophoretically through the channel formed by α-hemolysin.[115] That study suggested the polynucleotides thread through the pore. A subsequent report showed that, if a large macromolecule is attached to one end of the polynucleotide, the free end of the DNA can still enter into the pore and reduce the channel conductance, but the polymer cannot translocate.[116,117] These negatively charged "ball-and-chain" polymer constructs were used to corroborate the location of the limiting aperture in the α-hemolysin channel and its length[118] (Figure 5). Specifically, ball-and-chain

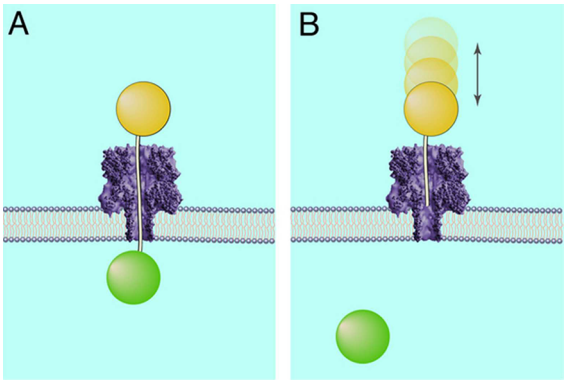

Figure 5. Determining detailed structural features of an ion channel using polymeric "molecular rulers". (A) Determining the length of the channel and (B) locating the limiting aperture in an ion channel.

polynucleotides that were too short to be driven into the narrowest constriction, where the electric field is greatest,[119] only transiently blocked the channel conductance.[118] Once the polymers exceeded a critical length, they blocked the pore conductance until the polarity of the applied potential was reversed. By adding the ball-and-chain polymers to either side of the membrane, the relative distances of the constriction in the pore from the two pore mouths were determined.[118] By adding a binding site on the free end of the polynucleotide for another macromolecule, the relative length of the α-hemolysin channel was also determined. In this case, a ball-and-chain polynucleotide of a given length was added to one side of the membrane and driven into the pore by the applied potential. The macromolecule that could bind to the free end of the polymer was added to the other side of the membrane, and the potential was repeatedly reversed. The polymer was ejected out of the pore from the side it entered only if it was shorter than the length of the pore. When the ball-and-chain polymer exceeded the pore length, the macromolecule was able to react with its binding site on the free end of the polymer and thereby cause the construct to be stuck in the pore indefinitely. These simple methods provide a means to estimate the locations of topographical features in a channel lumen and the length of a pore.

2.5. Selectivity

Perhaps the most stunning feature of ion channel function is the ability to exquisitely discriminate between different ions, including Na^+, K^+, Cl^-, and Ca^{2+}. When this capability goes awry, it often has significant health consequences, including cystic fibrosis[21,22](the most common lethal genetic disease among Caucasians[120]), epilepsy,[121,122] cardiac dysfunction,[14,16,17,20,123] and negative reactions to anesthetics (e.g., malignant hyperthermia[124]). The detailed molecular basis of ion channel selectivity is becoming fairly well understood,[44,125−127] perhaps because it is relatively easy to measure for channels reconstituted into planar lipid bilayer membranes.

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3. STATIC HIGH-RESOLUTION STRUCTURE

Although there is no substitution for determining and understanding the function of transporters, ion channels, and porins, simple electrophysiology experiments can only provide so much information about the topology and chemistry of a pore structure. High-resolution structures are imperative for a complete understanding of the various molecular mechanisms involved in ion selectivity, small-molecule transport, gating mechanisms, and binding sites as well as other various details associated with the protein's function.

Crystallography in all of its guises, whether derived from X-rays,[128] neutrons,[129] or electrons,[81] represents the gold standard of high-resolution structural analysis. Not surprisingly, the first protein crystal structure determination focused on large, easy to isolate and purify water-soluble proteins myogobin[130] and hemoglobin.[131] These studies, along with the 3-dimensional determination of DNA base-pairs,[132,133] provided an impressive foundation for the burgeoning field of molecular and structural biology. Despite intensive interest in the broader community, the first X-ray crystal structure of a membrane protein was not solved for another three decades (although electron microscopy produced a high-resolution crystal structure in 1975[134] vide infra) with the publication of the crystal structure of the photosynthetic reaction center from *Rhodopseudomonas viridis*.[70,135] This powerful work proved that membrane protein structure determination was possible; however, the rate at which new structures are solved severely lags that achieved for soluble proteins.[136,137] Currently there are 359 unique solved membrane protein structures,[138] and there is now at least one example from each of the major known families of ion channels.[4] The bottlenecks for membrane protein crystallography from its onset and still today remain primarily a challenge of expression and purification of a large amount of protein (i.e., hundreds of milligrams) and the subsequent crystallization.[81,139]

The relative paucity of channels in comparison to structures of soluble proteins is due to the significant challenges in the expression, purification, crystallization, and structure determination of integral membrane proteins.[140] These still remain nontrivial and challenging endeavors that require substantial time and resources. However, the rapid increase in the emergence of structures in the recent past could be attributed to developments in the technology and the experimental techniques that can be utilized to ultimately improve the structure solution process.[81,139,141]

If one were to probe for the bottlenecks unique to membrane protein structural biology, all signs would indicate that they lie upstream of the actual crystallography-related processes. The major hurdle is in the sample preparation stage. This can be divided into three principle areas: protein expression, protein purification, and crystal growth. Of these three steps, the protein-purification step is perhaps the best understood.

3.1. Protein Expression

The initial crystal structures of integral membrane proteins were obtained using protein purified from naturally abundant sources. Proteins purified using recombinant techniques are still in the minority in the ranks of membrane proteins that have been crystallized. This is in stark contrast to the structural elucidation of soluble proteins that have been purified using recombinant techniques. Overexpression methodologies that have been streamlined for soluble proteins do not fare well in membrane protein expression.[141] This is perhaps due to the difficulties related to recombinant membrane protein expression in foreign hosts. In general, during the course of expression, membrane proteins after transferring from the ribosome have to achieve their appropriate fold, and that protein must be targeted to and inserted into the membrane. The secretory machinery is responsible for the targeting and insertion, and during the course of protein overexpression that machinery is significantly burdened due to the expression of several intracellular proteins. This causes the expressed membrane proteins to form inclusion bodies. When eukaryotic proteins are expressed in a prokaryotic host, several factors can stymie the process. The most significant is the lack of glycosylation and other post-translational modification machinery in prokaryotic cells. The other problematic factor could be the differences between the membrane lipids of prokaryotic and eukaryotic cells. Despite these caveats, several eukaryotic integral membrane protein structures have been elucidated from proteins overexpressed in prokaryotic cells. The expression of several functional G-protein-coupled receptors in *E. coli* has been reported,[142,143] and a mammalian membrane-bound enzyme expressed in *E. coli* has been crystallized[144] and its structure determined.[145] The knowledge of how a membrane protein is synthesized in a cell is still poor, limiting the generalization of expression for further analysis.[81] The preparation of recombinant membrane proteins has been the predominate source of crystal structures since 2006.[81] One of them, prostaglandin H2 synthase-2 (COX-2), was expressed in insect cells,[146,147] while the others, monoamine oxidase-B and fatty acid amide hydrolase, were expressed in yeast[148] and bacteria,[145] respectively. It is interesting to note that none of these three proteins exhibit a very large hydrophobic surface area or are seen to be deeply inserted into the membrane. In this event, if the expression in prokaryotic cells yields unfavorable results, eukaryotic cells can be used to express the proteins. The expression systems that are utilized are yeast, insect, and mammalian cells. This hierarchy is largely determined by costs and the simplification of experimental design. The yeast expression system is favored because of the considerable manipulative genetic tools that are available and because they are relatively easy to grow. In particular, the methylotrophic yeast *Pichia pastoris* has been in focus for expression-related studies of membrane proteins.[149] This organism has a tightly regulated and potent high-level expression from the methanol oxidase promoter and can be grown to very high cell densities. For instances in which yeast expression systems do not suffice, it would be necessary to move into more complex organisms. Baculovirus-mediated expression in insect cells is now the most widely used technique.[150] Although this method of expression usually provides functional proteins, often the yields are not high and limit its applicability for further structural studies. Mammalian cell expression is perhaps the most promising of all of the protein expression avenues. They yield correctly folded proteins, and the yields can be optimized using an inducible expression system. This would be extremely useful for the production of membrane proteins that are toxic to the host organism (e.g., human proteins expressed in yeast). However, the associated costs and the less-than-trivial nature of the technique prevent it from being the method of choice.

3.2. Protein Purification

After the choice of the type of expression host employed and the optimization of the expression, the protein target must be

purified, using standard biochemical techniques. This typically requires using detergents to solubilize the membrane protein. The hydrophobic portions of the detergent molecules adsorb onto the hydrophobic surface of the protein, resulting in a protein−detergent complex that is stable and soluble in aqueous solvents and other cofactors such as substrates and ligands.[151] The choice of detergent is a critical issue to consider when designing protein-purification studies. There are dozens of different detergents used routinely in biochemistry and many more that are less well characterized but still potentially useful, with many novel detergents still under development.[152] Furthermore, mixtures of detergents are commonly used,[153] and nondetergent additives such as osmolytes (e.g., PEG) play critical roles in many cases.[143] Therefore, the size of the detergent parameter space is very large indeed. Because membrane proteins are not yet predictable in their interactions with detergents, it is impossible to identify the optimal detergents a priori. In general, a detergent should solubilize the target protein and preserve it in an unaggregated state. To achieve efficient purification, affinity tags are attached to the protein at either termini. The most common tags that are used are polyhistidine, maltose-binding protein (MBP), and glutathione-*S*-transferase.[154] For a successful crystallization outcome, the purity and homogeneity of the membrane protein are the most important parameters that need to be addressed. The formation of nonspecific aggregates needs to be avoided. Techniques that are useful to soluble proteins such as dynamic light scattering (DLS) are not useful for analyzing membrane proteins due to the presence of detergents, which can increase the heterogeneity of the dispersion. Gel-filtration and ultracentrifugation studies can shed light on the aggregation states of the protein. Recently, target ion channels have been tagged with a cleavable green fluorescent protein to aid the purification through size-exclusion chromatography,[155,156] which has become instrumental in high-throughput sequencing methods.

3.3. Crystallization

Another hurdle in membrane protein structure elucidation is protein crystallization. The resultant crystals must be ordered and diffract to an appreciable resolution in order to determine the structure. Many reviews have highlighted the crystallization techniques that have been developed.[140,157−160] There are two preferred techniques that are routinely used and have been validated by various research groups. They are either directly crystallizing the protein−detergent complex or introducing the protein into a lipid bilayer environment before growing crystals. In terms of statistics, the majority of X-ray crystal structures of integral membrane proteins have been determined using crystals grown directly from solutions of protein−detergent complexes. The most common method is by vapor diffusion. The lattice contacts comprise protein−protein interactions with detergent molecules interspersed between the protein molecules. The choice of the optimal detergent is a very time-consuming endeavor because it entails the physical characterization of the protein in the presence of a variety of detergents. Since the early years of membrane protein crystallization, choosing the right detergent has been the key to success. Well-ordered crystals of the photosynthetic reaction center from the purple bacterium *Rhodopseudomonas viridis* could only be grown using *N*,*N*-dimethyl dodecylamine-*n*-oxide as detergent.[70] Even use of the decyl homologue did not lead to crystals, and recent experiences confirm this observation. The cytochrome *c* oxidases provide illustrative examples. Crystallization attempts with the cytochrome *c* oxidase from bovine beef heart mitochondria continued for about 20 years, and crystals have been obtained in a number of different detergents; however, only the use of *n*-decyl-13-D-mahoside (C10-maltoside)—a mild, well-known detergent—has yielded well-ordered crystals. Cytochrome *c* oxidase from the soil bacterium *Paracoccus denitrificans* is another typical example. For the purification and crystallization of the four-subunit complex, only detergents of the maltoside-type can be used. All other detergents remove subunits III and IV, leaving an active complex consisting of subunits I and II. Only *n*-dodecyl-13-D-maltoside (C12-maltoside) leads to the formation of well-ordered crystals of the four-subunit oxidase as a complex with an Fv fragment. Recently, the catalytically active two-subunit complex could be crystallized, again with the help of an antibody Fv fragment in different detergents. Originally, crystals were grown using the C12-maltoside, but these diffracted to only about 8 Å resolution. Crystals grown with hexaethylene glycol monododecyl ether (C12E6) showed the same poor diffraction quality. With the Cl0-maltoside, no crystals could be obtained at all. Recently, the Cll-maltoside became also commercially available. Crystals grown in this detergent usually diffract to higher resolutions (Ostermeier, Harrenga, Ermler, Michel, from ref 158). Similar crystals can be grown with cyclohexylhexyl-[3-D-mahoside] (CYMAL-6), but not with cyclohexylpentyl-13-D-maltoside (CYMAL-5). These results show that even small chemical differences in the detergent can cause essential differences in the crystallization behavior of these detergent−membrane protein complexes. The conclusion has to be drawn that more efforts should be put into screening various detergents for crystallization than into the variation of other parameters. A major problem may be the high cost of many detergents. The optimal way to cope with this hindrance is to purify the protein using a rather inexpensive detergent such as Triton X-100 or *N*,*N*-dimethyldodecylamine-*N*-oxide and then to exchange the detergent prior to the crystallization attempts. One should keep in mind that it may be difficult to control the completeness of the detergent exchange. Other agents can also aid in the crystallization process. For example, addition of an antibody fragment can increase the probability of producing crystals because it enhances the polar surface area.[161] Despite the numerous successes associated with the crystallization of protein−detergent complexes, some proteins require insertion into a lipid bilayer. This was first achieved when diffracting crystals of bacteriorhodopsin were grown in the presence of a lipidic cubic phase.[162] The lipidic cubic mesophases form gel-like materials containing continuous bilayer structures, arranged so as to form topologically distinct lipid and aqueous regions.[163,164] Proteins are added to preformed lipidic cubic phases, upon which they partition into the bilayer. Precipitating agents can then be added to induce crystal growth "in cubo".[165,166] A modified technique that combines the elements of both the lipidic cubic phase method and the protein−detergent complex method has recently been described.[167] The method employs bicelles, which are small bilayer disks that dissolve in aqueous solutions much as micelles do, but offer an environment more like a native biological membrane.[168] One should keep in mind that the challenges of sample preparation are present for all ion channel measurements. Crystallographers have led the development through sheer numbers. However, these lessons can be applied to many of the techniques in this review.

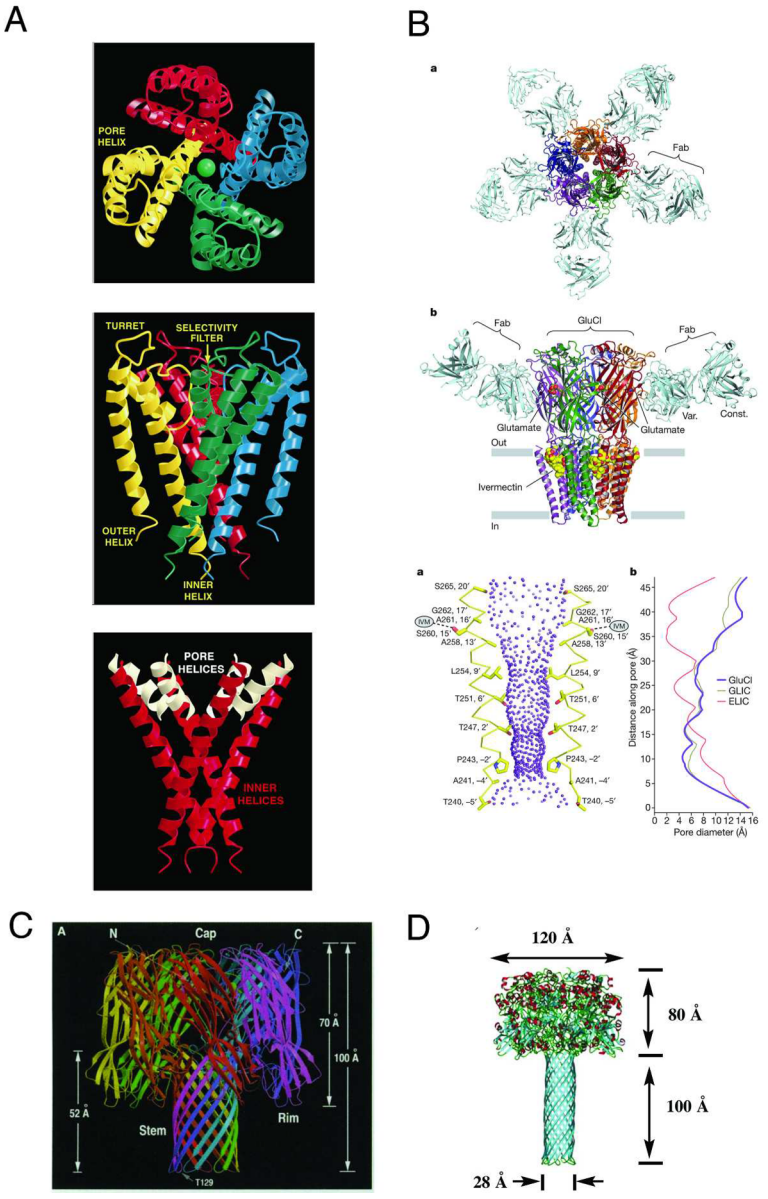

Figure 6. X-ray crystallography has provide the highest resolution and most diverse set of structures solved to date. (A) Structure of the first K^+ ion channel KsvA[44] has led to many new and more complicated structures including (B) glutamate-gated chloride channel α[183] (C) a pore forming toxin αHL.[28] and when crystals cannot be formed computer models can adapt known structures to produce hypothetical structures to arbitrarily high-precision (D) $(PA63)_7$ model based on αHL.[413] (A) Reprinted with permission from ref 44. Copyright 1998 The American Association for the Advancement of Science. (B) Reprinted with permission from ref 183. Copyright 2011 Macmillan Publishers Ltd.: Nature (C) Reprinted with permission from ref 28. Copyright 1996 National Academy of Sciences. (D) Reprinted with permission from ref 413. Copyright 2004 Adenine Press.

3.4. Channel Structures

Despite the difficulties described above, structures of several new ion channels are being solved each year.[138] The earliest and still highest profile structures were of K^+ channels[44,169,170] (Figure 6A). These studies finally shed light on a special class of proteins that are ubiquitous throughout the proteome[171] and play a central role in regulating transmembrane potentials. The single most interesting discovery in this work was the structure of the selectivity filter of the channel. The crystal structure of the bacterial KscA channel has proven to be the crowning achievement of ion channel research.[44] This work demonstrated unequivocally that the K^+ selectivity filter is formed by a narrow constriction 12 Å long that is constrained such that K^+ but not Na^+ ions are coordinated in the constriction when the ions are dehydrated.[44] The filter works in part because the chelating residues are held in a precise geometrical arrangement such that the energy to dehydrate K^+ but not Na^+ is compensated by direct interactions of the channel with the ion. Importantly, these structures developed our physical understanding of selective ion channels,[172] guided the characterization and analysis of many other ion-selective channels,[44,173−182] and have been extended to very complicated proteins with up to 16 transmembrane helixes[183] (Figure 6B). A more complete list can be fount at Stephen White's database Web site.[138] Beyond simply increasing our understanding of how ion channels function, these and related studies are allowing ion channels to rival G-protein coupled receptors (GPCRs) as therapeutic targets for drug intervention.[184−187]

Beyond developing the molecular and atomistic basis for ion channel functional mechanisms, high-resolution structures have

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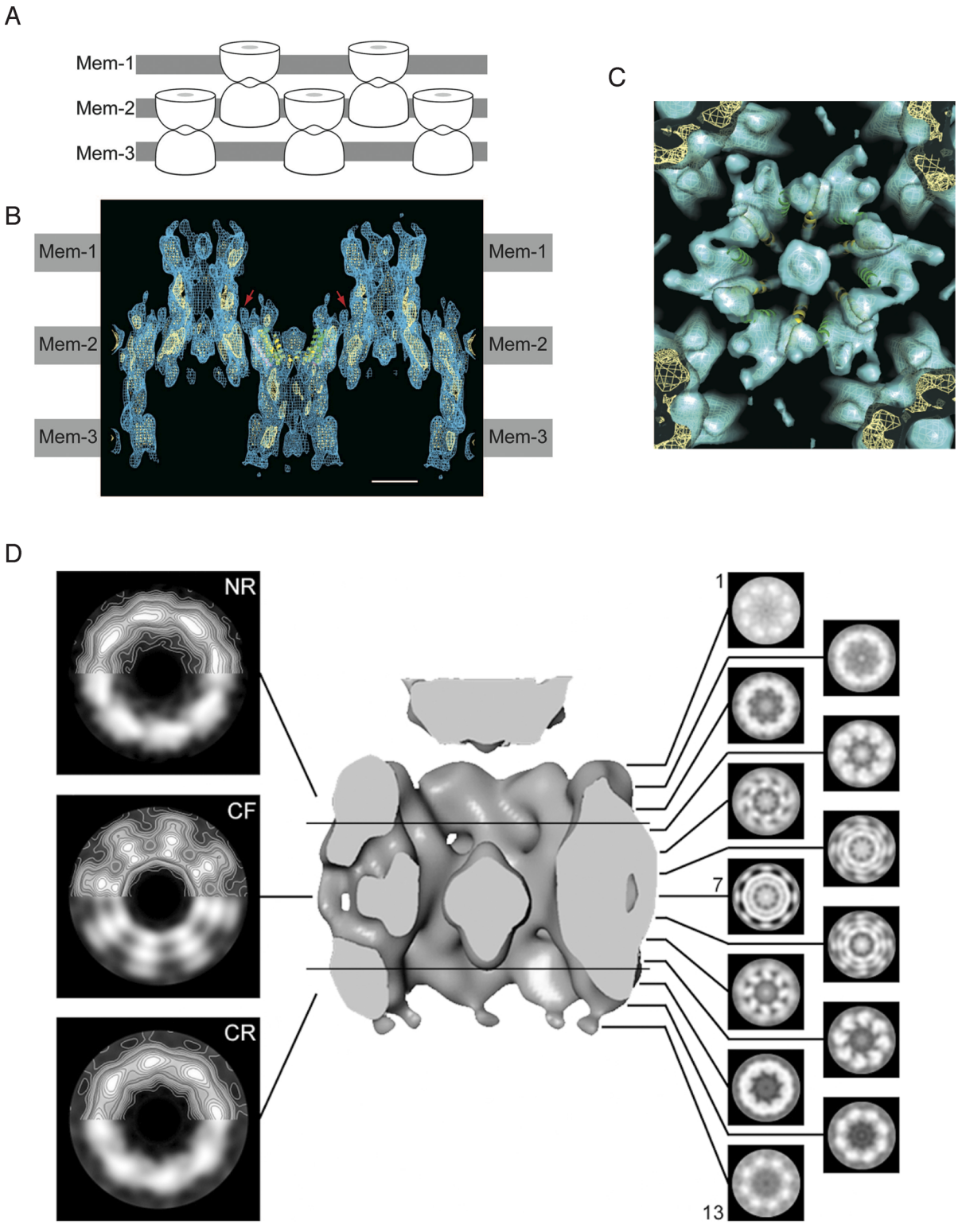

Figure 7. Electron microscopy operates in two modes: diffraction and direct imaging. (A) Diffraction off of 2D surfaces can be used to generate detailed nearly atomistic structures such as this result from connexin[199] and (B) direct imaging of a frozen nuclear-pore complex shows tomographic slices with resolution of better than 6 nm.[195] (A) Reprinted with permission from ref 199. Copyright 2007 National Academy of Sciences. (B) Reprinted with permission from ref 195. Copyright 2003 Elsevier.

made inroads to studying disease states. In one such example, the crystal structures of several purported disease mutants of an isolated domain of a ryanodine receptor (RyRs) result in large Ca^{2+} release channels.[188] There are nearly 200 mutations identified in the RyR1 isoform that are associated in many disease states linked to Ca^{2+} signaling including malignant hyperthermia,[189] 150 mutations in the RyR2 isoform linked to catechol-aminergic polymorphic ventricular tachycardia,[190] arrhythmogenic right ventricular dysplasia type 2, and idiopathic ventricular fibrillation.[191] The size of the underlying

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protein (~2 MDa) and staggering number of known mutations linked to different diseases suggest that there may be many different structural changes that affect the protein's function in different ways. Indeed this is the case. Some mutations in the RyR1 ion form suggests that phosphorylation and disease mutations occur at the same interface of the protein—one mutation in particular alters the thermal stability of the protein—or that the mutations change the surface properties of the binding sites and interdomain salt bridges.[188] Although this work is promising, a great deal of work remains to tie these domains experimentally to the channel essentially tying the results to the physiological mode the diseases that are caused by such mutations.

Some ion channels with β-barrel pore structures have had their structures determined using X-ray crystallography. These are typically pore-forming toxins like *S. aureus* α-hemolysin[28] (Figure 6C). Many of these structures evolved as lethal toxins and are responsible for many of the symptomatic effects of acute infectious diseases.[29] These β-barrel structures are also implicated in antibiotic-resistant Mycobacteria (tuberculosis and leprosy are caused by bacteria in this genus), which have low-permeability outer membranes. Channels in these membranes such as MspA from *Mycobacteria smegmatis* form extended β-barrel pores for the transport of nutrients into the cell.[192] High-resolution structures of such toxins should aid in the development of therapeutic agents against them.

4. ELECTRON MICROSCOPY

The first structures of moderately high-resolution membrane proteins were solved by electron microscopic (EM) techniques.[134] Despite EM's early entrance into solving structures of membrane proteins, as a practical technique, it lagged crystallography and NMR spectroscopy as a go-to technology for the determination of protein structure and was only included recently in the archival Protein Databank.[193] In fact, EM can easily operate in two modes: direct imaging[194−198] and diffraction.[134,199−201] The resolution of EM ranges from ~2 Å, high enough to see fundamental kinks and structures in a peptide to ~100 Å, which is limited to looking at larger globlar "structures" and aggregation. The true advantage of EM lies in its relatively simple sample preparation and the minimal material required to make measurements.

As electron microscopy becomes more popular, more ion channel structures will likely become available. One such effort is the examination of connexin26, a channel that is associated with hearing loss. Electron diffraction was used to solve the structures of human connexin26 at high resolution.[199] Further confirmation of the functional behavior of these channels have been confirmed with single-molecule electrophysiology[202] and comparison of open and gated configurations with a combination of crystallography and cryo-EM[203] (Figure 7A). Like connexin, cholera toxin interacts with multiple membranes and has been studied directly with cryo-EM (Figure 7 B).[204,205] Several successful Ca^{2+} channels have been studied,[206] RyR1[207] as well as a host of K^+ channels: KvAP,[208] KscA (shaker b),[209] MloK1,[210] Kir2.1,[211] and nonselective TRPA1 a pain and inflammatory response receptor.[212] Electron microscopy can also be used to validate—or call into question—crystal structures. For instance, a crystal structure for the cystic fibrisis transmembrane channel was developed,[213] refining the structure from a known structural homologue, the *Staphylococcus aureus* Sav1866 channel.[214] Studies such as this demonstrate the power of developing structures from multiple lines of research to broaden and extend the impact of the difficult to obtain crystal structures.

5. DYNAMIC HIGH-RESOLUTION STRUCTURE

Although a crystal structure offers the highest resolution for membrane protein structure, the results provide only a snapshot in time with no temporal information, and only a fleeting possibility to see a protein in provably functional forms. However, a truly functional protein is dynamic by nature and requires more complete study than is made possible from static observations.[215] To counter these limitations, NMR offers a complementary method for atomistic-resolution structural biology. At the opposite end of the energetic spectrum, NMR measures the energy difference in nuclear spin states when the sample is placed in a large, static magnetic field. With the utilization of high magnetic fields and modern pulse protocols, each atom in a protein produces a unique signal,[76,216] which allows for precise atomically resolved structures with resolution on the same order and precision as that determined by X-ray crystallography. Recent reviews highlight the significant advances in the field and will be discussed briefly.[216−222]

5.1. Methods in NMR: Generating Meaningful Signals with Complex Samples

NMR comes in two distinct forms: solution-phase and solid-state NMR. There are two key differences between the two techniques: sample-preparation schemes and pulse sequences used to generate meaningful signals. Regardless of the technique, all NMR experiments are performed by placing a sample in a high magnetic field. This field serves two purposes. First the magnetic field aligns the dipoles of all spin centers (in protein samples these are typically ^1H, ^2H, ^{13}C, and ^{15}N), and it separates the energies of each spin-state of these atoms into distinct and quantifiable energy levels (typically in the microwave range). NMR scientists have long been known as champions for complex acronyms describing the pulse protocols that define an experiment. Those most commonly found in protein structure experiments go by the names such as TOCSY,[223] TROSY,[224,225] NOESY,[226,227] PISEMA,[228] PISEMO,[229] and many others.[230] Regardless of the methodology chosen, all NMR experiments basically follow a four-step protocol: preparation, evolution, mixing, and detection.[75] The preparation phase consists of a delay time where thermal equilibrium is achieved, followed by *rf* pulses to create a coherent system (i.e., the spins of interest are aligned in phase transverse to the magnetic field). In the evolution phase, the coherence of the spins evolves according to their relaxation times and Hamiltonian operator of the spin. Following the evolution phase, the spins are mixed with a series of *rf* pulses and delay intervals to transfer coherence among the spins of interest. Finally, in the detection period, a free-induction decay (FID) is caused by the spin coherence relaxing back to thermal equilibrium.[231] These generalized timing schemes can be developed in arbitrarily large dimensions—often with 3 and 4 dimensional techniques being extensively applied to protein structure experiments.[232] Structures are then determined through a combination of *J* coupling, through bond resonances, and nuclear Overhauser effect (NOE), through space resonances. Often it is necessary to couple both bond and space techniques to develop a three-dimensional structure.

5.2. Solution NMR

The correlation time problem[233] has been known since at least 1921. In NMR spectroscopy, the physical manifestation of the

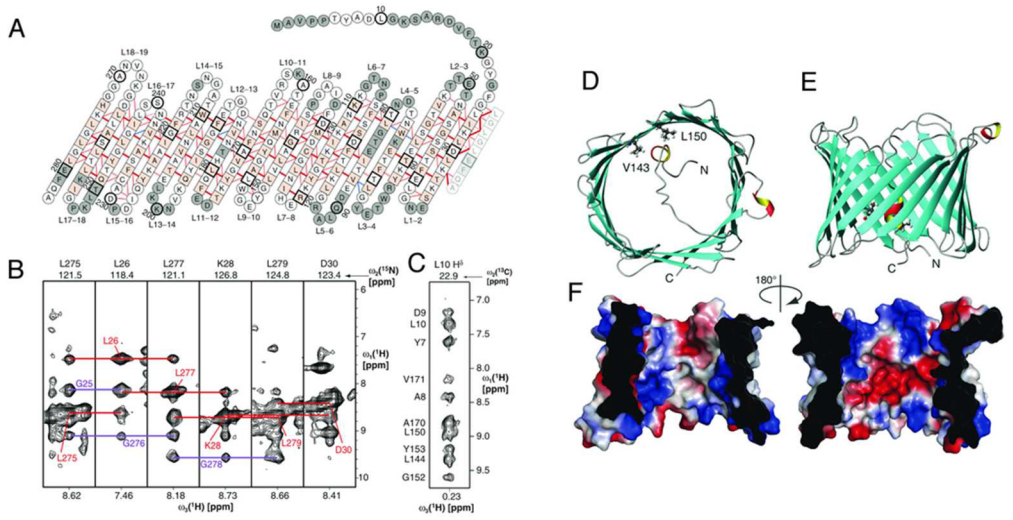

Figure 8. Architecture of VDAC-1 arranged according to higher structures. Experimentally observed NOE contacts are highlighted with red and blue lines (A). Sections from a 3D [^{1}H, ^{1}H]-NOESY ^{15}N-TROSY experiment that defines the NOE contacts between β-sheets 1 and 19 (B). Section from a 3D [^{1}H, ^{1}H]-NOESY ^{13}C-HMQC (heteronuclear multiple-quantum coherence) taken at the position of a methyl group in Leu.[10] These spectra were used to calculate the solution structure of VDAC-1 (D) and (E) and produce a van der Waals surface of the protein in its most probable mean configuration (F). Reprinted with permission from ref 248. Copyright 2008 The American Association for the Advancement of Science.

correlation arises when the analyte becomes large such that the reorientation of the molecule is slow with respect to the spin relaxation rates. Slow tumbling fails to average out the anisotropic interactions resulting in broad resonances and ultimately unusable spectra.[221] The spin correlation time was a major limiting factor for decades in NMR spectroscopy. However, a number of protein-labeling strategies[234−237] and pulse protocols have largely overcome this limitation.[224,226,238−244] Regardless of the protocols developed to reduce the peak broadening and other associated artifacts in solution, NMR spectroscopy specialized sample-preparation schemes are necessary to achieve maximal resolution. As most membrane proteins are insoluble in water or are likely to denature if directly solubilized, it is necessary to dissolve the protein with detergents. Sample preparation for solution NMR has progressed in two complementary ways. Reducing the volume of solution around the protein through micelles and reverse micelles[245,246] and reducing the viscosity to effectively increase the tumbling rate.[245−247] Figure 8 demonstrates a particularly impressive example of the resolution that can be achieved in detergent micelles through 3D [^{1}H, ^{1}H]-NOESY ^{15}N-TROSY pulse sequences to study VDAC-1.[248] The crystal structure of the voltage-dependent anion channel, VDAC-1, was also determined.[176,178] Interestingly, these VDAC studies offer a comparison between the two technologies. Both technologies determined nearly identical structure of the 19-stranded β-barrel core of the protein.[249] The difference highlights the relative strengths of each measurement. The crystal structure was able to determine the voltage-sensing region of the *N*-terminal segment bound inside the pore, whereas NMR did not clearly resolve this feature—perhaps due to experimental conditions enhancing the mobility of this segment of the protein. Conversely, in the NMR experiment the protein activity was tested in situ through the addition of nicotinamide adenine dinucleotide, which regulates gating.[249] Spectral shifts can then be correlated to changes in the binding site of this and other small molecules as they interact with the protein. This is a unique and critical feature of dynamic measurement techniques, which are completely unobtainable with crystallographic techniques.

High-quality NMR spectra of proteins have been obtained up to 900 kDa,[244,250] and these techniques have been applied to several channels including KscA,[251] OmpX,[252] OmpG,[253] human VDAC-1 (Figure 8),[177,248] the influenza B proton channel,[254] and a wide range of ion channel fragments such as the voltage-sensing region of KvaP,[255] the proapoptidic BAX[256] to a host of fragments of the influenza M2 channel.[257−260] The M2 channel is particularly important as antiviral drugs based on adamantane bind to the pore and inhibit ion conductance. Mutant strains of M2 have diminished binding efficiencies of these drugs leading to drug resistant strains of the flu.[261]

5.3. Solid-State NMR

Although solution NMR spectroscopy has produced a large number of membrane protein structures, often these determined structures are focused on the structurally conserved extra membranous segments that can more easily be solubilized with detergents while retaining their functional form. Of course, the ideal environment for the high-resolution structural determination is the membrane of the organism from which the protein originates. This is not a practical goal for most (or really any) membrane protein targets. There are, however, several bilayer constructs that can mimic a cellular membrane, host membrane proteins, and support NMR experiments. These include bicelles (aka nanodisks), multilamellar vesicles, unilamellar vesicles,[168,262−267] and solid supported membranes deposited on layers of glass.[268,269]

Once a suitable membrane mimic is chosen, the challenge for the NMR experiment is to produce meaningful signals with peaks narrow enough to eliminate spectral overlap. The discovery that dipole−dipole interactions are anisotropic provided the basis for distance and angular measurements between different spin centers in a crystalline lattice.[270] There is an intrinsic appeal to this methodology due to the relative ease of calculating distances and angles based upon dipoles rather than other interactions of nuclear spins.[271−273] This tremendously useful approach through separated local fields has

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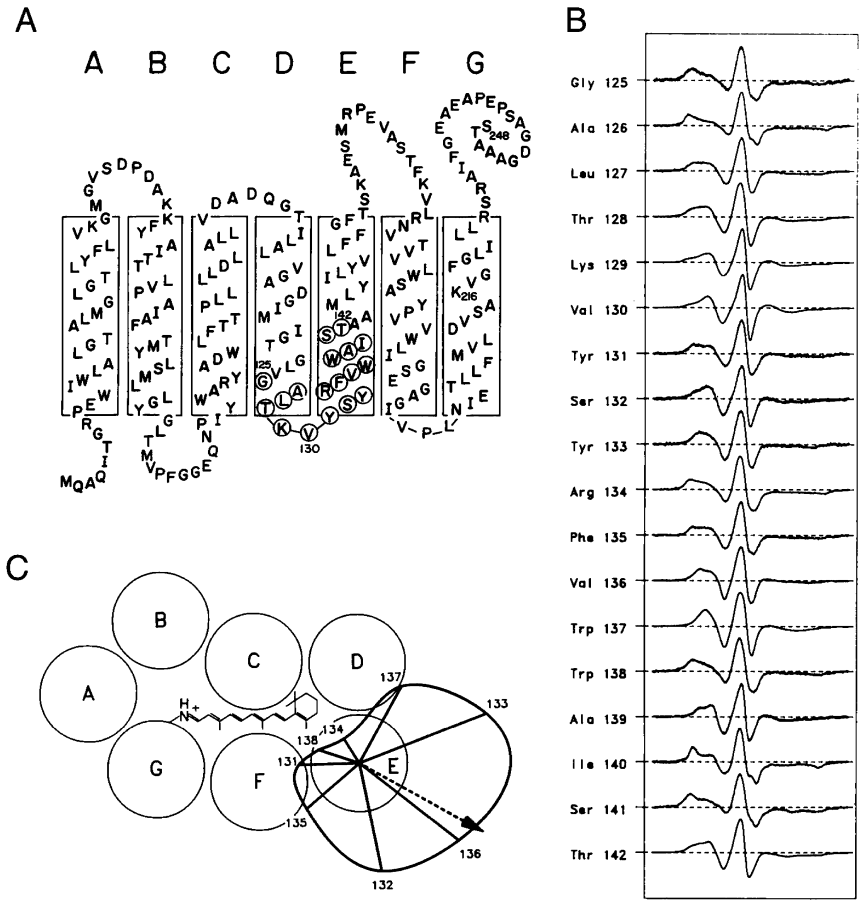

Figure 9. EPR spectroscopies can determine through site-directed spin labeling the position of specific residues within a membrane.[305] (A) Location of mutations in bacteriorhodopsin used to test the accessibility of oxygen and chromium oxalate to the spin-labels. (B) EPR spectra were used to determine the motion of each of the spin labels. (D) The accessibility of oxygen to helix E is superimposed on a model of the protein. Reprinted with permission from ref 305. Copyright 1990 The American Association for the Advancement of Science.

become instrumental in structural studies of membrane proteins.[216,274] The separated local field techniques allow solution and solid-state NMR to be unified through the use of magnetically aligned nanodisks.[262,275] Separated local field methods are particularly useful in protein structure determination with uniformly ^{15}N labeled proteins. The amines in a protein's backbone are well-separated by the two carbon atoms, and the adjacent ^{1}H can be used as the detected spin to improve the sensitivity.[229] High-resolution spectra can also be obtained with site-directed ^{13}C enrichment.[276] One particular advantage of NMR over complementary structural techniques is the ability to measure the conformational dynamics of the protein over a wide range of time scales from picoseconds to hours.[277]

5.4. Structures for Disease-Related Proteins

As with solution NMR, solid-state NMR (ssNMR) has focused first on small peptides in part to validate the technique (and because the spectra are less complex). ssNMR solved the first structure of gramicidin-A,[278] a pore-forming antibacterial agent in channel form.[279−281] Because gramicidin is formed transiently as a dimer where each monomer diffuses freely in opposing monolayers, these channels do not crystallize. Other structures have been solved for peptides related to M2 channel[282−284] and complete with amantadine binding sites.[285] The flexibility of ssNMR shows clear structural rearrangements of the KscA-Kv1.3 channel when exposed to scorpion toxin kaliotoxin.[286] This result in particular demonstrates that the flexibility of the K^+ channel and the toxin are responsible for the high binding constants between the two proteins. Park and colleagues took advantage of the complementary nature of ssNMR and solution NMR to determine the structure of the transmembrane helix from residues 8−25 of HIV-1 Virus Protein "u" (Vpu).[287] Subsequent electrophysiology experiments confirmed that this helix oligomerizes into a pore-forming complex with a ~96 pS conductance and hypothesized that the protein is associated with budding of virus from infected cells. NMR has also been used to study protein misfolding with extensive studies of amyloid fibers related to Alzheimer's disease[288] with particular focus on residues 1−42,[289] which are thought to form channels in membranes.[290] In addition to direct protein structure determination, ssNMR can also investigate the lipids surrounding proteins. In particular, the method has been used to test the role of tryptophan-rich proteins and their ability to form pores in membranes. One study used a pore with a known crystal structure, the maltoporin from *Salmonella typhimurium*, a classic trimeric β-barrel,[5] to study the interactions between the tryptophan and tyrosine-rich pore with the bilayer membrane.[291] This study suggests a strong role of aromatic residues with the headgroups of lipid bilayers and could aid in the understanding of other pore-forming toxins.

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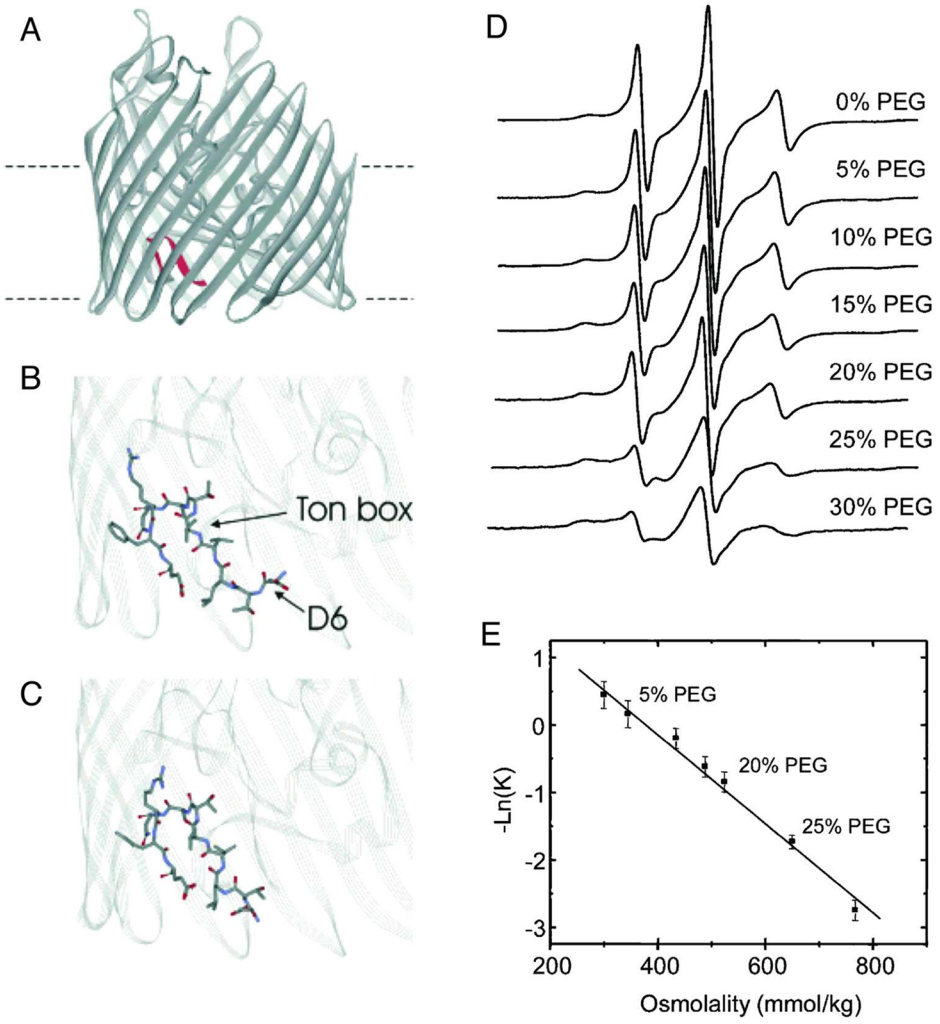

Figure 10. EPR spectroscopy provides evidence that osmolytes can adversely effect the crystallization process. (A–C) The crystal structure from BtuB[414] is shown highlighting the Ton box, a vitamin B_{12} binding site, within the protein interior. (D) EPR spectra obtained as a function of poly(ethylene glycol) concentration (E) demonstrate a dehydration of the pore forcing the protein into its "docked" state.[328]

6. EPR SPECTROSCOPY

Electron paramagnetic resonance spectroscopy (EPR) is an analogous technique to NMR spectroscopy. Unlike NMR spectroscopy, EPR measures resonances in electron spins and requires samples to be paramagnetic (i.e., there must be an unpaired spin in the protein). For many proteins with metal centers, the technique is a natural extension to the NMR measurements, with specific detection of the catalytic centers of the protein, but with many ion channels, the measurement requires spin-labels engineered in at key sites in the protein.[292,293] EPR can be run in two modes, the classical continuous wave mode, which is a simple adsorption measurement made by scanning either the magnetic field or the *rf* field,[294] or with pulsed techniques, which operate like pulsed NMR methods.[295]

EPR methods take advantage of a long history of chemical and bioengineering approaches that incorporate spin labels into membranes[293] or proteins.[296−298] Thus, using site-directed spin labeling (SDSL) transforms EPR into a method to readily measure conformational changes in proteins. Although EPR uses extensive labeling to determine structural characteristics of proteins, it has two main advantages over NMR: the sensitivity is much higher, requiring only 1 pmol of protein, and there is no inherent difficulty in measuring spins from proteins and complexes of any size.[299] Like NMR spectroscopy, EPR has the capability to measure dynamics and distances between labeled residues within a protein.[297,299−303] As new computational tools that can generate high-resolution structures from sparse EPR data are developed, EPR is poised to become an even more crucial tool in the structural biologist's arsenal.[304]

EPR was the first major technique to successfully probe the solvent accessibility of a helix structure in a membrane protein directly. SDSL was used to label bacteriorhodopsin in consecutive positions along an α-helix. The lineshapes, due to motion, motional anisotropy, environmental polarity, and hydrogen bonding, which in practice are too complicated to directly probe structure, were manipulated with soluble probes with variable size and polarity to determine structural features of the protein (Figure 9).[305]

EPR spectroscopy played an instrumental role in determining the molecular mechanism of K^+ transport by the ubiquitous K^+ family of ion channels. All K^+ channels known display a common structure with highly conserved toxin-binding sites and selectivity filters.[306] Crystal structures only offer a static picture, and to date no structure of the fully deactivated state has been determined, although there are compelling structures

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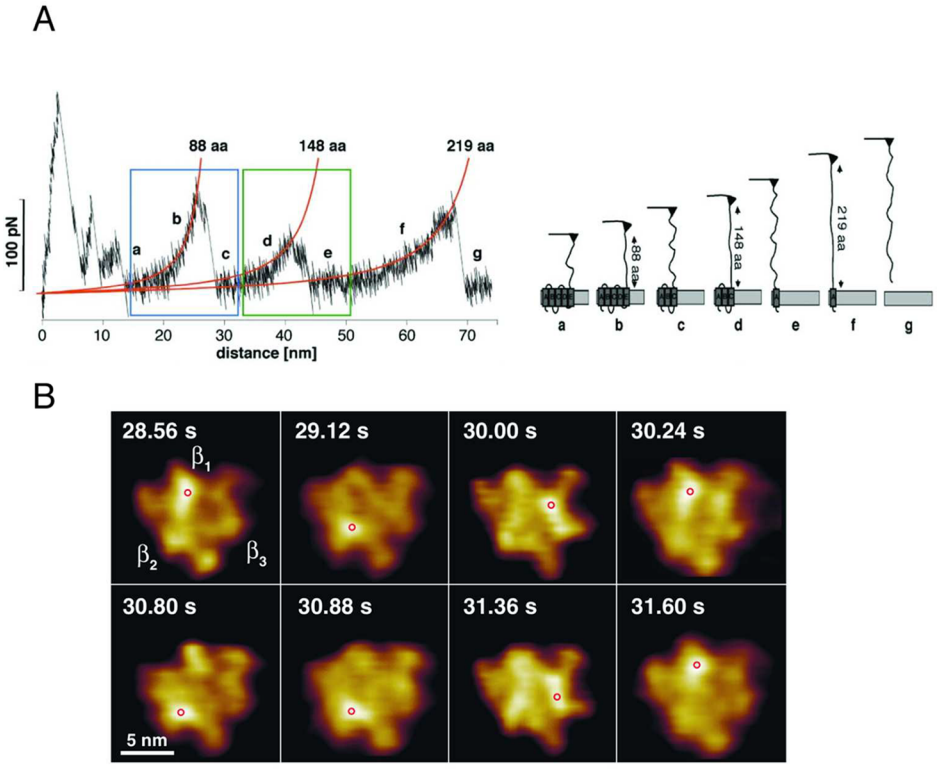

Figure 11. Atomic force microscopy provides two distinct measurement modalities. (A) Force spectroscopy can measure the force required to unfold and remove an isolated bacteriorhodopsin from from a membrane.[415] (B) Imaging across a surface with high-density protein coverage can provide detailed images in the plane of the membrane; with high scant rates protein motility can be measured.[345] (A) Reprinted with permission from ref 415. Copyright 2004 Elsevier. (B) Reprinted with permission from ref 345. Copyright 2001 National Academy of Sciences.

from molecular dynamic (MD) simulations.[307] The ability of EPR to measure conformational changes in biomimetic systems provides the experimental link between the crystal structure and the purported structures from MD computations. SDSL experiments immediately provided links between open and closed states of the protein, by observing dynamic results of the transmembrane helices of the channel[308−311] as well as monitoring the limited reactivity of cystine centers buried within the channel region.[312,313] At the most ambitious, SDSL experiments can be used to refine crystal structures by observing small-molecule (i.e., oxygen and nickel ethylenediaminediacetic acid) collision rates at labeled sites within the protein.[314] Beyond simply confirming or refining the crystallography results, and perhaps adding some detail on open and closed states, SDSL was used to observe interactions between the channel and solvent and lipid.[315−318] These results are significant in the study of ion channel diseases in part because of the indication that single-site mutations (a common genetic link to channelopathies) can alter the way that membrane-spanning segments interact with lipid bilayers.[316] EPR has also provided a link between the voltage-sensing and pore domains of KvAP.[319] The work by Cuello and colleges demonstrates one critical limitation of all of the techniques discussed so far: no technology can reliably or effectively apply an electrostatic potential across the membrane. These techniques have also been applied to other types of channels, such as sodium channels[320] and mechano-sensitive channels.[321]

These methods have also been used to probe the resting-state conformation of ABC transporter MabA.[322,323] ABC transporters are responsible for nutrient uptake and are thought to be involved in multidrug resistance in bacteria.[323,324] They have been instrumental in characterizing the effects of substrates and ligands on conformational changes of the outer-membrane transporter TonB,[325−327] and it has even been suggested that osmolytes used in crystallization buffers may lock the channel into the closed state through dehydration (Figure 10).[328] The ability to monitor longer range interactions has made EPR a valuable technique for the determination of the molecular mechanism of anthrax infection. The channel-forming subunit PA63 forms heptameric[329] or octameric[330] pores, which bind two ~90 kDa anthrax toxins, lethal factor and edema factor, and catalyze their transport across endosomal membranes. The crystal structure of the heptameric prepore was previously determined,[331] but the atomic structure of the pore has been elusive.[30] EPR has been used to determine conformational changes as the β-barrel forms, suggesting that a phenylalinine residue at position 427 is implicated in pore formation[332] and transport across the membrane.[333] The most detailed examination of the interaction with the *N*-terminal fragment of lethal factor with the heptameric PA63 pore suggests that there are three distinct interaction sites on and within the pore that are instrumental in the transport of lethal factor into cells.[334] Although these studies purport the translocation of full-length lethal factor through the 1.2 nm diameter PA63 channel pore,[98,103] they generally use only about one-third (≈the first 250 residues) of the *N*-terminal domain, which is known to be a globular segment of the protein.[335] Other evidence (unpublished) suggests that, under semiphysiological conditions, the interaction between lethal factor and the PA63 channel is too strong for lethal factor to be translocated through the pore and released into the cytosol.

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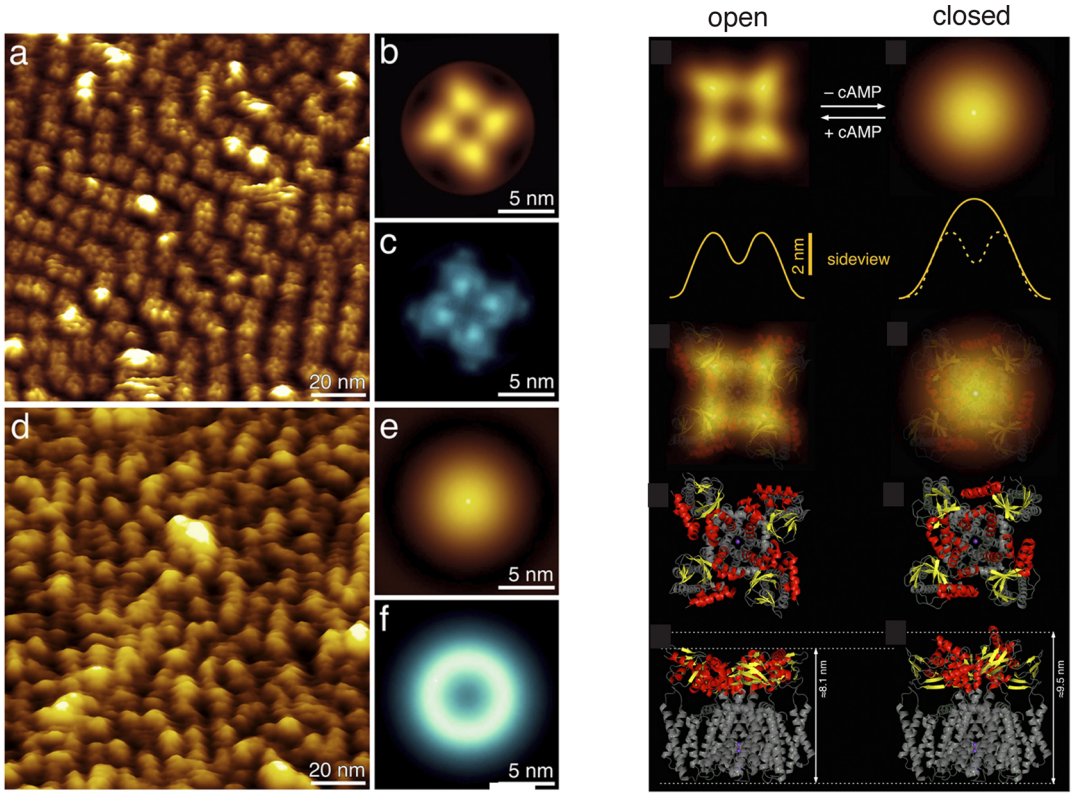

Figure 12. High-resolution images can observe the gating properties of a cAMP-regulated ion channel. Subtle shifts in the transmembrane segments can be resolved and compared to models generated from crystal structures to illuminate functional mechanics of the protein. Reprinted with permission from ref 362. Copyright 2011 National Academy of Sciences.

7. EMERGING TECHNIQUES

7.1. Atomic Force Microscopy

The invention of scanning probe microscopy[336] ushered in a new era in the visualization and characterization of surfaces.[337] The technology rapidly became a popular technique for quick investigation of the structure and aggregation and polymers with atomic resolution.[338] The technique found early application to study biomolecules at high resolution by investigating isolated DNA molecules,[339] crystals of amino acids,[340] and phase transitions in Langmuir–Blodgett films.[341−343]

AFM operates differently than almost all other analytical techniques. Instead of using probes of light, subatomic particles, or ions, AFM uses a mesoscale mechanical probe, usually a sharpened silicon nitride tip mounted on a cantilever with a known spring constant. The instruments can be run in two distinct modes, imaging (where the probe is rastered in the plane of the surface, providing resolution that approaches that of EM measurements)[79,344,345] and a force spectroscopy mode (where a single protein can be denatured mechanically while observing the force that it requires to remove the protein from a membrane) (Figure 11A).[346−350] Recent developments in AFM instrumentation have allowed for video frame rate images (Figure 11B)[345] to be recorded, thereby providing kinetic information about catalytic rates in F_1-ATPase.[351] Because the AFM can be utilized in solution of bilayers spontaneously spread on Si or mica surfaces, the sample preparation is among the least intensive of the structural techniques. Typically vesicles with protein incorporated in them are allowed to fuse onto a hydrophilic, atomically smooth substrate. This spontaneously creates lipid bilayer incorporated proteins without further treatment or modification. Beyond fusing vesicles, it is also possible to fuse membrane fragments directly from bacterial membranes.[352,353] Further, ionic or electronic transport can be measured simultaneously to image if the membrane is built over a cavity,[354,355] tethered to an electrode surface,[356−358] or whole cells are imaged in a patch-clamp apparatus.[359]

The potential for moderately high-resolution and dynamic structural changes makes AFM an attractive method to study ion channels. Mari and colleagues recently observed pH-induced structural changes in the OmpG.[360] These results confirmed that the purported gating mechanism in which one or more of the extracellular loops of the protein collapses into the channel entrance under physiological conditions *in native lipid membranes*. With sufficiently high resolution, small changes such as detecting a putative Ca^{2+} binding site on an AQP1 can be achieved.[361] Gating transitions were observed in MlotiK1, a cyclic nucleotide regulated K^+ channel.[362] The AFM results suggest that the protein undergoes a transition that increases the height above the membrane by about 1.4 nm as the protein gates. These results are compared favorably with the crystal structure of the open channel,[363] and with a model created from the cAMP-unbound CNB domain.[364] MlotiK1 is structurally analogous to many other K^+ channels such as KscA, dShaker, MtHK, etc.[365] AFM imaging detected structural changes of MlotiK1 and an R348A mutant with and without cAMP. By comparing the 2D AFM images with models generated from crystal structures of the transmembrane segment of the protein alone,[363,364] a mechanism was deduced for the gating of the full protein (figure 12). Although these imaging techniques are limited to 2D topography, it is possible

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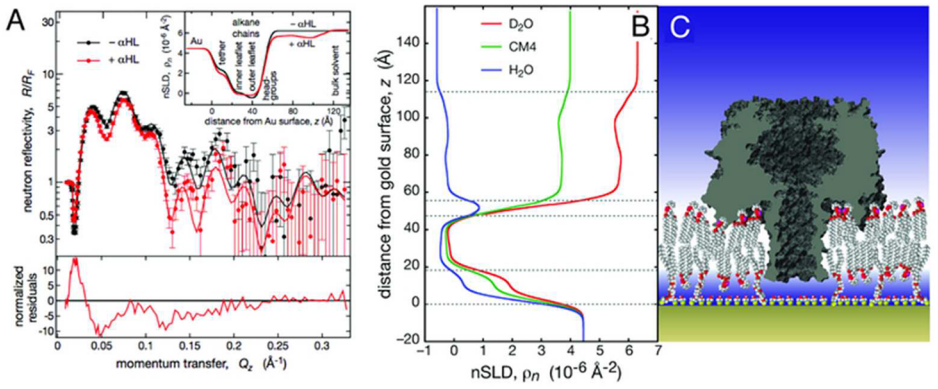

Figure 13. Neutron reflectometry of α-hemolysin in a tethered bilayer reveals the protein−lipid interactions to a resolution of 1.1 Å. (A) Reflectivity curves recorded in D_2O before (black) and after (red) the formation of αHL channels show subtle but clear shifts in the reflectometry data. (B) Calculated scattering length density (SLD) curves for under three solvent contrasts and refined with the theoretical SLD for αHL suggest that the protein intimately interacts with the lipid headgroups. (C) A scaled illustration of the system analyzed. Reprinted with permission from ref 408. Copyright 2009 Cell Press (Elsevier).

to observe distinct differences between aquaporins with a high level of sequence homology.[361]

AFM studies are playing an increasing role in the structural mechanism for disease through the study of toxic β-sheet ion channels.[366] Many amyloid β-peptides are known to interact with membranes, forming plaques, fibrils, or pores in the membrane.[367−373] These studies suggest that Alzheimer's disease pathology arises from a globular aggregation of amyloid β-protein rather than from the fibers often considered the likely culprit for the disease.

7.2. Neutron-Based Techniques

Neutron-based analytical methods have a long history in protein analysis. At the onset, neutrons were considered important as a complement to X-ray crystallography. Neutrons interact exclusively with atomic nuclei rather than the electron cloud. Thus, rather than being most sensitive to larger atoms, neutrons have a more complicated selectivity.[78] The single most important property of neutrons for structural biology arises from this complexity. Because they interact with the nucleus, neutrons have different scattering lengths for different isotopes. In particular, of the atoms most prevalent in biology, neutrons have a very large sensitivity to the difference between hydrogen and deuterium. X-rays in contrast cannot detect hydrogen in any meaningful way.[129,374,375] However, the crystal needs to be considerably larger for neutron studies than for X-ray analysis.[78]

To study ion channels in biomimetic interfaces, two neutron-based techniques have emerged as promising avenues of study: small-angle neutron scattering (SANS) and neutron reflectometry and diffraction. Although SANS experiments for characterizing ion channels are in their infancy, the stability of vesicles and ability to incorporate peptides[376−379] and proteins such as colicin[380] suggest the method will prove useful. Coupled with more established methods such as fluorescence spectroscopy,[381−384] function can also be probed, although membrane potentials cannot be controlled. It remains to be seen how SANS coupled with fluorescent assays can probe structure and function of ion channels and how they relate to disease, but the promise of these techniques remains high. In the meantime SANS is especially sensitive to protein aggregation and can detect and characterize aggregation or clustering of ion channels, such as the mechanosensitive channel MscL, mediated by vesicles.[385]

Neutron reflectometry has clear and obvious application for the study of ion channel-based diseases. The challenge for a reflectometry experiment is to produce a stable bilayer membrane for direct reflectometry or a hydrated stack of bilayers for diffraction experiments that do not change appreciably over the duration of an experiment, which can last for several days of continuous measurement. Several options exist for this demanding condition and have been reviewed recently.[357,386] The goal for the membrane is to incorporate protein in high enough density to detect contrast between either the protein and the membrane or protein and the solution layer adjacent to the membrane. Additionally, it is important that the lipids retain their lateral fluidity in order to accommodate the protein's functional movement. Multilayers of lipid accomplish this with the separation of each leaflet with the hydration layers associated with the headgroups.[387−394] These interfaces provide enough structural stability and thickness of ordered layers diffraction patterns. The lipidic environment is known to have a profound impact on the orientation of the voltage-sensing region of potassium voltage-dependent potassium channel (KvAP).[395,396] Neutron diffraction was used to reconstruct scattering length density profiles for the bilayer with S1−S4 voltage-sensing domains of the KvAP.[397] The contrast between ^1H and ^2H was used here to determine structural changes of the lipid−protein interface while estimating the distribution of water throughout the system. The protein causes the membrane to thin by only ~3 Å, suggesting that the voltage-sensing domain evolved to minimize the energetic perturbations of the bilayer, leading to highly efficient gating. Because the voltage-sensing domain does not extend beyond the membrane, the detection and characterization is primarily dependent on secondary structural changes (i.e., membrane shifts, redistribution of water, etc.). To achieve a large enough signal for competent analysis diffraction techniques from multilamellar surfaces were required. For large proteins that extend well beyond the membrane, such as bacterial exotoxins (e.g., α-hemolysin, PA63, or cholera toxin), reflectometry can be performed on single bilayers[398] and the protein contribution to the signal can be either deciphered directly or inferred from changes in the lipid bilayers. Cholera toxin was shown to alter lipid packing on a lipid monolayer surface.[399] For bilayer experiments, the surface-optimized tethered bilayer membrane (tBLM) was developed.[358,400−404]

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These membranes use a synthetic thiolipid with a poly(ethylene glycol) spacer between 4 and 9 or greater repeat units long. Bilayers created from this interface can facilitate single ion channel measurements[405,406] and produce membranes that are stable for protein insertion for several months.[407] By combining electrical measurements to define the function of αHL with NR signals refined with the high-resolution crystal structure, the position of functional α-hemolysin pores was determined with an uncertainty of only 1.1 Å (figure 13).[408] Other techniques for attaching proteins directly to electrode surfaces[409] or through engineered his_6-tags[410] also show promise for the functional incorporation of other membrane proteins of various complexity. This method may also prove useful for studying the interactions between ion channels and agonists, toxins, or therapeutic agents (i.e., where they bind, binding constant, and stoichiometry).

8. CONCLUSION

Ion channels play a central role in many diseases. To develop treatments for these diseases, it is critical that the molecular mechanisms related to the structure and function of such channels are understood in great detail. Take cardiac diseases as a high-profile example. Many heart-related diseases originate from ion channels malfunctioning and failing to generate the required action potentials needed for muscle contraction at the appropriate time. This is an intricate balance between K^+, Na^+, and Ca^{2+} channels relaying timing signals throughout the organ.[411] Structural and functional studies of ion channels are and will continue to be used to guide the development of new drugs. It is clear that the specificity of a drug to its target protein will seriously reduce unexpected and undesired side effects.[412] Today this is principally done with X-ray crystallography and NMR spectroscopy. New technologies are providing complementary information to our current understanding of molecular biology, and although they will never displace the current technology, they will certainly shed light on the processes necessary to produce high-quality protein samples for atomistic resolution and produce new rapid screening technologies to more quickly test drug candidates.

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Notes

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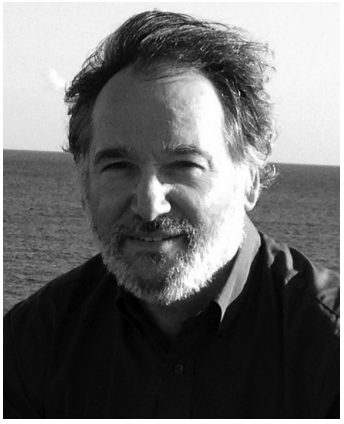

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