The 54th Annual Meeting and Symposium of the Society of General Physiologists

Structures and Mechanisms of Channels and Transport Proteins

(ORGANIZED BY JANOS K. LANYI and CHRIS MILLER)

The general features underlying the membrane protein-catalyzed transfer of material across cell membranes have emerged from increasingly sophisticated "black box" studies on protein function (defined broadly), but mechanistic understanding of membrane protein function has been slower to emerge—except, maybe, for ion channels. A major limitation has been the lack of high resolution structures that could serve to guide the design and constrain the interpretation of experimental results. This dearth of structural information arose for two reasons: first, it is difficult to purify sufficient amounts of protein to begin crystallization; and second, even when this barrier has been crossed, it is difficult to obtain well-ordered membrane protein crystals that allow for structure determination. These barriers are eroding (slowly) because of advances in bacterial protein expression and methodological advances in 2- and 3-D crystallization of membrane proteins, and the number of high resolution membrane protein structures has increased dramatically over the last three years, or so. These new protein structures form the anatomic basis for new studies on membrane protein physiology; and they highlight the importance of high resolution physiological studies for extracting mechanistic information from protein structures.

The recent advances in membrane protein structure determination, and the interdependence of molecular anatomy and physiology, was the focus of the 54th Annual Meeting of the Society of General Physiologists, which took place in Woods Hole, MA, September 7–9, 2000. Janos K. Lanyi (University of California, Irvine) and Chris Miller (Brandeis University) organized the symposium on Structures and Mechanisms of Channels and Transport Proteins, which highlighted the progress in structure determination that has taken place over the 15 years since Johann Deisenhofer and Hartmuth Michel solved the structure of the bacterial reaction center. With more than 200 participants, and 75 abstracts covering a broad range of topics, the meeting was lively and the major issues were put in perspective for all.

Most advances in membrane protein structure determination continue to come from studies on proteins of bacterial or organellar origin, which seem to be less temperamental when it comes to crystallization and structure determination than their eukaryotic plasma membrane counterparts. Bacterial membrane proteins segregate into two classes (Fig. 1): (1) proteins from the outer membrane in which the membrane-spanning domains fold as β-barrels; and (2) proteins from the inner membrane in which the membrane-spanning domains fold as α-helical bundles. Bacteria also secrete a wide variety of membrane-active toxins in which the membrane-penetrating domains may fold into either β-barrels or α-helical structures. Thus, bacterial proteins constitute a rich environment for mechanistic studies on membrane protein structure and function, as highlighted throughout the Symposium, where two thirds of the presentations were on bacterial proteins.

Bacterial outer membrane proteins are of interest not only because of their β-barrel structure, but also because they have to pass through the inner membrane, and through the periplasmic space, before they insert into the outer membrane. The underlying molecular gymnastics remain unknown; but, it may share similarities with those observed for the secreted bacterial toxins, such as α-hemolysin, which undergoes a major conformational change as it adsorbs at the target cell membrane. The cytotoxic channel is a heptamer that is assembled after adsorption, which leads to assembly/insertion of the bilayer-spanning β-barrel. Once formed, the heptamer is stable in SDS, and the electrophysiological properties of α-hemolysin channels are remarkably well-defined, with a single major conductance state. The pore diameter is ~11 Å, which is large enough to allow cyclodextrins to enter, and bind into, the pore. H. Bayley (Texas A&M University) showed how cyclodextrin binding is signaled by discrete conductance changes (decreases). Moreover, cyclodextrins in the pore can bind a wide variety of guest compounds—to cause a further conductance decrease, which serves as an electrophysiological fingerprint for guest compound binding in the cyclodextrin-adapted α-hemolysin pore. The pore's β-barrel structure and insertion pattern make the pore permissive with respect to modifications of the

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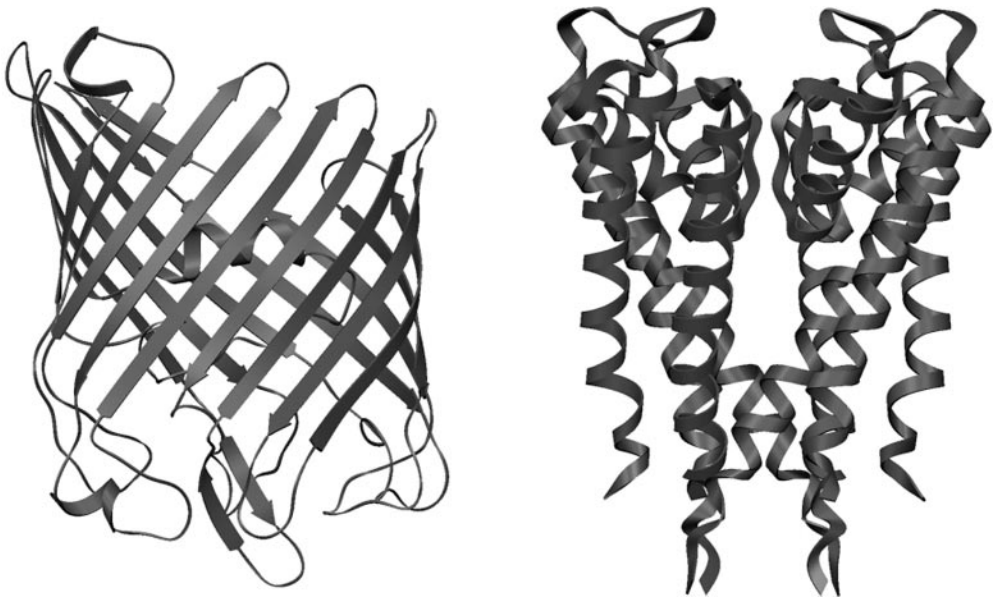

FIGURE 1. Membrane protein structures as ribbon diagrams. (Left) An outer membrane β-barrel, OmpF (Cowan, S.W., T. Schirmer, G. Rümmel, M. Steient, R. Gosh, R.A. Pauptit, J.N. Jansonius, and J.P. Rosenbusch. 1992. *Nature.* 358:727), PDB accession code 2OMF, resolution 2.4 Å. Right: a plasma membrane α-helical bundle, KcsA (Doyle, D.A., J.M. Cabral, R.A. Pfuetzner, A. Kuo, J.M. Gulbis, S.L. Cohen, B.T. Chait, and R. MacKinnon. 1998. *Science.* 280:69), PDB accession code 1Bl8, resolution 3.2 Å. We thank Dr. C. Lima (Weill Medical College of Cornell University, New York, NY) for assistance with the figure, which was produced using the program SETOR (Evans, S. 1993. *J. Mol. Graph.* 11:134).

pore-lining residues, such as to allow for the design of pores that discriminate among different cyclodextrins, which are selective for different guest compounds. Thus, it is possible to design α-hemolysins that can be used as sensors for a wide variety of compounds. To understand the α-hemolysin–cyclodextrin interactions better, the pore dimensions were probed using SH-reactive polyethyleneglycerols. The results of these studies are summarized in an article by Movileanu et al. in this issue of *The Journal.*

The physiological importance of the outer membrane, and of outer membrane proteins was highlighted by S.K. Buchanan (Birkbeck College, UK) and W. Welte (Universität Konstanz, Germany), who reviewed the principles of bacterial solute movement and described two new structures involved in Fe^{3+} transfer across the outer membrane of *Escherichia coli*: a ferric enterobactin receptor (FepA); and a ferrichrome receptor (FhuA). FepA and FhuA are 22-stranded β-barrels. Their structures differ in important ways from previous outer membrane protein structures, the diffusion channels (or porins) that form large water-filled pores, because the barrel pores in FepA and FhuA are plugged by protein domains that mediate the selective, energy-dependent transport of Fe^{3+} into the cell. The porins mediate the (electro)diffusive movement of small solutes between the extracellular solution and the periplasmic space; but, even porins exhibit (modest) selectivity among different solutes (Welte). The "general diffusion porins" (e.g., OmpF [Fig. 1, left] and PhoE) are 16-stranded β-barrels in which the ion selectivity is based on valence. The "sugar porins" are 18-stranded β-barrels that can discriminate among related carbohydrates, e.g.: sucrose and maltose, in Sucrose porin (ScrY) and Maltoporin (LamB), respectively. The molecular basis for the sugar selectivity is a series of aromatic residues along the inner pore lining that form a path for sugar binding and transport. The need for several sugar porins arises because sucrose and maltose have different "shapes" (and sucrose cannot pass through LamB). The ability to discriminate among different sugars results from differential sugar binding in the narrowest part of the pore.

FepA and FhuA mediate an active, TonB-dependent transfer of iron across the outer membrane, where TonB spans the periplasmic space to physically couple FepA and FhuA, and other outer membrane proteins, to an energy source provided by the protonmotive force across the inner membrane. The domain within the barrel lumen forms the binding site for chelates of Fe^{3+} with either enterobactin (in the case of FepA) or ferrichrome (in the case of FhuA). When the Fe^{3+}-chelates bind at the extracellular end of the intrabarrel domain, they trigger a conformational change at the periplasmic end, which allows TonB to recognize the substrate-loaded receptor and enables the chelate to move through plugged barrel. The formation of the TonB–FepA (or TonB–FhuA) complex and the ensuing input of energy reduces the siderophore affinity for Fe^{3+}, which eventually causes the Fe^{3+} to be released to periplasmic proteins (e.g., FepB) that pass it onto ATP-dependent plasma membrane transporters. Overall, Buchanan and

Welte provided a marvelous picture of the intricacies of bacterial Fe^{3+} uptake. Moreover, they illustrated a common theme of the Symposium—namely, the interplay between studies on structure and function. The impact of a new structure depends on the amount of preexisting genetic and physiologic data that can guide mechanistic interpretations. The structure also allows for new approaches to understanding function because it, together with the preexisting information, allows for the design of experiments to test better defined questions.

α-Helical bundle structures were the main topic of the Symposium. D.C. Rees (California Institute of Technology) noted that 2001 is the 50th anniversary of the α-helix, and described the structure of the high conductance mechanosensitive channel (MscL) from *Mycobacterium tuberculosis*. MscL is gated (opened) simply by applying lateral tension to the plasma membrane. The activity–tension curves can be shifted by numerous mutations in the *E. coli* channel; but, the gating mechanism remains enigmatic, in part because the single-channel conductance steps are ~2 nS in 0.1 M KCl, indicating that channel activation (opening) is associated with a large change in pore diameter (and channel structure). Indeed, the shape (steepness) of the relation between membrane tension and channel probability indicates that the pore cross-sectional area change associated with channel opening is ~650 $Å^2$. The structure of a closed channel state was obtained after an exhaustive search for MscL orthologues that form stable, well-diffracting crystals. The channel is a pentamer; each monomer has two membrane-spanning α-helices, an inner (TM1) and an outer (TM2), which cross at a 40° angle such that each TM2 has contacts with two TM1 and each TM1 has contacts with two TM1 and two TM2 helices. In addition, a helical bundle projects from the cytoplasmic end of the structure to form a structure that is reminiscent of the so-called "hanging basket," which is seen also in other channels (see the next two paragraphs). When aligning the *E. coli* and *M. tuberculosis* sequences, the change-in-function mutations tend to be distributed throughout the structure with a tendency to cluster at TM1-TM1 contacts; but the detailed gating mechanism remains enigmatic.

Not all structures are solved by X-ray diffraction. N. Unwin (Medical Research Council, Cambridge, UK) described the structure of the nicotinic acetylcholine receptor (nAChR) as deduced by electron microscopy of tubular receptor crystals. Using a total of ~800,000 images, the current resolution is at ~4 Å, which is sufficient to deduce the major structural changes that occur when the channel is activated by acetylcholine (ACh) binding. The structure shows two putative ACh binding sites, at the α/γ and α/δ subunit interfaces, and ACh binding causes a rotation/tilt of the subunits relative to each other, which alters the orientation of a bend in the pore-lining M2 helices, from being toward pore axis (in the closed state) to a more radial position (in the ACh-bound, presumably open state). Access to the putative ACh binding sites is through a β-sheet lined path, and one can trace an α-helix from the binding site down to the membrane-spanning domain. In the narrow part of the ACh-free, presumably closed structure, the M2-lined pore is ~3.4 Å in diameter and 10 Å long. In the ACh-bound, presumably open structure, the pore diameter is ~2 Å wider. Moreover, by aligning the sequence to the structure, Unwin concluded that the ACh-induced rotation of M2 will change the pore lining from being predominantly hydrophobic in the ACh-free conformation to being more polar in the ACh-bound conformation. A final feature of the structure is the presence of a hanging basket at the cytoplasmic end of the structure, which is present whether ACh was bound or not. Ion access to the pore is through fenestrations in the link between the membrane-spanning domain and the basket domain, which is much more complex than we normally depict it!

A comparable structure was presented by N. Grigorieff (Brandeis University) based on electron micrographs and image reconstruction of single *Shaker* potassium channels. Single, detergent-solubilized *Shaker* channels were visualized in random orientations. The resulting pictures were classified into different views and, for each view, the final image was obtained as the average of >200 pictures. Combining the different images, a final model emerged at a resolution of ~20 Å. The structure shows two large domains that are connected by four stalks. The larger domain has the dimensions that would be expected for the membrane-spanning domain of the *Shaker* channel; the dimensions of the smaller domain (the hanging basket) were consistent with it being composed of the T1 domains from each subunit. Assuming that the native structure in the membrane is similar, access to the intracellular pore entrance will again be through the four "windows" defined by the domains and the four stalks that connect them, which is remarkably similar to the situation for the nAChR.

The power of electron microscopy for solving membrane protein structures was reemphasized by Peter Agre (Johns Hopkins University School of Medicine), who described a 3.8-Å resolution structure of aquaporin 1 (AQP1). Though lipid bilayers are permeable to H_2O, AQP1 forms the major path for H_2O movement across the red cell membrane. AQP1 is remarkably selective for H_2O, as neither monovalent anions nor cations, or even H^+, can permeate through the pore. The AQP1 amino acid sequence has six potential membrane-spanning segments; the two halves of the sequence result from gene duplication of a common an-

cestor, such that the two halves have opposite topology with respect to the bilayer. The structure is a tetramer, and reveals a pore through each monomer, where the lining in the central section is quite asymmetric: one side of the pore is polar, being lined by two asparagine residues; the other is nonpolar, being lined by hydrophobic residues. The cross-sectional area is sufficient to allow an H_2O molecule to pass the narrow central region, where it would be stabilized by hydrogen bonds to the two pore-lining asparagines. This organization would ensure that the pore was impermeable to anions, which would not be solvated by the asparagines. The cation impermeability could result because two α-helices (one from each half of the subunit) are organized such that their NH_2-terminal ends point toward the central region, which would disfavor cation binding/permeation due to electrostatic repulsion between the α-helix and a cation in the pore. The impermeability for H^+ could arise from the same mechanism; but, H^+ permeation also would be reduced if the two asparagine carbonyl oxygens could form hydrogen bonds to both hydrogens in an H_2O molecule in the central part of the pore, which would disrupt the hydrogen bond network that underlies the jumping of H^+ from H_3O^+ to H_2O.

D. Fu (University of California, San Francisco) described the structure of another member of the aquaporin family, the bacterial glycerol transporter GlpF. GlpF catalyzes the selective movement of glycerol across bacterial membranes. The rate of glycerol movement is close to the diffusion limit. Nevertheless, the pore is effectively impermeable not only to ions, but also to H_2O. The structure was solved to 2.2 Å by X-ray analysis, and showed an overall similarity to the AQP1 structure. Again, the pore-lining was amphipathic, with one side being nonpolar and the other being lined by backbone atoms and polar residues. Two glycerol molecules, separated by a H_2O molecule, are seen in the narrow part of the pore, where they form hydrogen bonds with three groups on the polar side of the pore. This organization allows for favorable binding of the amphipathic glycerol, even though there is no evidence for saturation of the glycerol flux up to 500 mM glycerol. Moreover, the organization of the polar groups is such that they would not interact favorably with H_2O: the H_2O molecule between the glycerol molecules in the pore forms hydrogen bonds to the glycerols. Thus, the structure provides insights into GlpF's impermeability to H_2O (in the absence of glycerol).

Another new structure reported at the meeting was that of a chloride channel from *E. coli*, EriC, which is homologous to the ClC family of vertebrate anion channels. J.A. Mindell (Brandeis University) presented the analysis of electron microscopic studies on 2-D crystals of EriC, which revealed a dimeric channel structure with evidence for (H_2O-filled) low density regions that most likely are the membrane-spanning pores. Detailed analysis of the electron density suggests that the pores might have kinks along their path; but, detailed information on the ion permeation pathways and their relationship to the individual subunits awaits a 3-D structure.

Considering the prevailing dearth of high resolution membrane protein structures, the respiratory chain complexes of mitochondria and aerobic bacteria stand out by virtue of the (relatively) large number of known structures. This unique situation could be a consequence of the very high protein/lipid ratio in the inner mitochondrial membrane, which might promote the evolution of protein–protein contacts that could stabilize the crystals. But, even so, progress may be slow because of the time (years) it may take to refine the crystallization conditions to the point where a high resolution structure can be determined. H. Michel (Max-Planck-Institute für Biophysik, Frankfurt, Germany) described a 2.2-Å structure of fumarate reductase from *Wolinella succinogenes*, for which the first crystals were obtained some 12 yr earlier. The structure illustrates an important feature of proteins involved in redox reactions, namely that the prosthetic groups in the structure provide strong hints about the underlying mechanisms. But, even so, the details may be difficult to extract, as Michel described for the case of H^+ pumping by cytochrome *c* oxidase in which a reaction scheme could be deduced only through detailed kinetic studies and electrostatic model calculations based on the 3-D structure.

The workhorse of membrane proteins is bacteriorhodopsin from *Halobacterium halobium*, which recently was crystallized using lipids that form bicontinuous cubic phases. As described by H. Luecke (University of California, Irvine), even then the structure was difficult to ascertain because there is hemihedral twinning in the crystals. Twinning means that a single crystal is composed of two separate subdomains, which can be superimposed in space after rotation of 180°, such that the diffraction pattern results from the summation of contributions from the two components of the twinned crystal. Taking the twinning into account, the X-ray structure is remarkably similar to the structure that previously was deduced by R. Henderson and co-workers based on electron microscopy of 2-D crystals. The presence of twinning means that one cannot use the full data set for analysis; nevertheless, the current resolution is at 1.5 Å, which is sufficient to visualize an extensive network of hydrogen-bonded H_2O through the crystal. It also is possible to discern a number of lipid molecules that serve as a glue between the individual bacteriorhodopsin molecules. The crystal form supports a relatively normal photocycle, and it is possible to follow the

photocycle using a combination of site-directed mutagenesis and low temperature quench experiments. The retinal 13-trans→cis photoisomerization causes an extensive reorganization of the H_2O network, which is initiated by a 1.5-Å movement of the retinal 13-methyl group. This movement causes the methyl group to "bump into" Trp^{182} and thereby trigger a propagated conformational change from the extracellular toward the cytoplasmic side, which causes the release of a H^+ to the extracellular solution followed by the reprotonation of the retinal Schiff base from the cytoplasmic side. The H^+ is transferred from, and to, the retinal Schiff base by transient hydrogen-bonded networks.

Some important membrane proteins, or protein domains, remain recalcitrant. The structure of the F_1 domain of the mitochondrial ATP synthetase, for example, has been solved by J.E. Walker and co-workers; but, the membrane-spanning F_O domain is known only at low resolution. R.H. Fillingame (University of Wisconsin Medical School) showed how it is possible to infer essential details about the structure of a membrane-spanning protein without having crystals. In fortunate circumstances one can obtain structural information from high resolution NMR on proteins in micelles, oriented bilayers, or organic solvents. Fillingame used the latter system to deduce a model for the *c* subunit of F_O, which was used to design cross-linking experiments to probe the subunit stoichiometry and the contacts between the *a* and *c* subunits of F_O.

A different approach to structure determination without crystals was described by H.R. Kaback (University of California, Los Angeles), who summarized an astounding amount of data based on chemical modification and cross-linking experiments in the *E. coli* lac permease. The lac permease is composed of 427 amino acids, but only six are irreplaceable, which means that one can use mutants with one or two cysteines to probe accessibility, functional significance, and cross-linking ability. The latter results form the basis for a set of distance constraints, similar to those obtained in 2-D nuclear magnetic resonance (2-D NMR) experiments, which together with the constraints obtained from the accessibility measurements can be used to obtain a structure at a resolution of ~4 Å. One surprising conclusion from this work is that the lac permease is a highly flexible molecule, presumably reflecting the extent of the conformational changes that occur during the H^+-driven lactose transport.

A third approach to structure elucidation without crystals was presented by F. Bezanilla (University of California, Los Angeles), who summarized strategies for probing the voltage-dependent movement of the S4 segments and its relation to potassium channel activation. Gating current measurements show that the "voltage sensors" (the S4 segments) can move without causing channel activation, and that at least four equivalent charges in each subunit cross the membrane when the channels activate. Systematic replacement of the arginines and lysines in the S4 segment with histidines allows for more detailed probing of the S4 movement by monitoring the protonation/deprotonation of the histidines from the extra- and intracellular solutions, which becomes a measure of accessibility. Again, at least four charged residues can cross the membrane. The movement of the S4 segments relative to each other was probed using lanthanide-based fluorescence energy transfer. The results showed remarkably small changes in energy transfer, indicating that there is little relative movement of the S4 segments (~1 Å). The results also tend to exclude any significant out-of-plane motion. The most parsimonious interpretation of the data is that S4 undergoes a simple rotation, and that the voltage dependence arises because there are deep crevices into the structure from both surfaces, such that the rotation moves the S4 charges from one crevice to the other, which will cause an effective movement across the membrane.

The importance of low resolution structures was amply illustrated by D.L. Stokes (New York University School of Medicine) and G.A. Scarborough (University of North Carolina), who spoke about the Ca^{2+}-ATPase from the sarcoplasmic reticulum (SR) and the P-type H^+-ATPase from *Neurospora*, respectively. Both Stokes and Scarborough used the recent high resolution structure of the SR Ca^{2+}-ATPase, obtained by C. Toyoshima and colleagues (*Nature.* 405:647, 2000), to guide a reexamination of previous results. Previous domain motions, which had been deduced from electron microscopy or proteolysis, could be mapped onto the high resolution structure. The X-ray structure could be overlaid on the structure of the E_1 form of the ATPase, which has a high Ca^{2+} affinity. But the E_2 form, which has a low Ca^{2+} affinity, could not be overlaid unless one imposed a significant movement of the P domain of the high resolution structure relative to the rest of the molecule; i.e., the combination of a related high resolution structure with previous lower resolution structures, as well as functional data, provides new insights into the ATP-driven substrate movement. Even so, the domain movement does not provide insights into how substrate accessibility to the transmembrane domain is controlled; even a high resolution structure may not be sufficient to provide mechanistic details about the transport event.

Another approach to understand membrane protein structure and function is to completely remove the membrane-spanning segment and determine the structure of the resulting construct that, hopefully, should mimic the structure in the intact molecule—an assumption that can be tested, for example, by comparing

ligand affinities for the native molecule and the construct. E. Gouaux (Columbia University, College of Physicians and Surgeons) presented results on the ligand-binding domains of the ionotropic glutamate receptors. The amputated subunits were constructed by eliminating the membrane-spanning segments and fusing the two domains that form the ligand binding site. The resulting constructs crystallize as tetramers, which turn out to be dimers of dimers with an agonist binding site in each dimer. Their structures have been solved to better than 2 Å. The ligand binding site is in a cleft between the two domains in each subunit, and ligand binding triggers a significant motion of the domains relative to each other. The extent of the cleft closure varied as a function of the ligand affinity (binding energy), and there are similarities between the structural changes that occur when agonists and antagonists bind. Overall, the results provide strong support for the notion that ligand binding–dependent closed→open transitions are driven by the difference in ligand binding energy between the closed and open state. The two ligand binding sites interact strongly, and ligand binding to both cause shifts in the relative positions of residues at the tetramer surface that would face the bilayer in the intact receptor. Given the extent of the observed shifts, binding of glutamate could cause a 10-Å shift in the transmembrane helices of the receptor. The actual movement could be less in the intact receptor because of the "resistance" imposed by the reorganization of the transmembrane segments; but, the results suggest that channel activation is associated with substantial changes in the organization of the membrane-spanning domain.

The molecular motions associated with channel activation were discussed further by E. Perozo (University of Virginia), who described the results of electron spin resonance (ESR) studies on the *Streptomyces lividans* potassium channel, KcsA (Fig. 1, right). ESR spectroscopy has many advantages, one of them being the modest sample volume that is needed for getting the spectra. De novo structure assignment remains difficult, but see the article by Cortes et al. in the February issue of *The Journal* (Vol. 117:165–180). But given a structure (and lots of mutagenesis and covalent labeling with nitroxide probes), it is possible to deduce the range of motions that take place during channel closed↔open transitions, which in the present case are controlled by H^+ binding at the cytoplasmic side of the channel. ESR spectroscopy provides three different kinds of information: (1) the spectral shape provides information about the (rate of) motion; (2) the spectral coupling between different probes provides information about the distance between the probes; and (3) the spectral quenching by lipid- or water-soluble probes provides information about exposure to the bilayer or aqueous solution. When evaluating the distance information, one should keep in mind that the nitroxide probes have a size similar to tryptophan sidechains. Therefore, any particular distance measurement will provide limited information about the motion of, say, a helix backbone. But, given a sufficiently large set of distance measurements, it is possible to deduce a structure using distance-dependent algorithms similar to those used in 2-D NMR experiments. Similarly, given a reference structure and two sets of distance measurements, it is possible to deduce the extent of any movement associated with pH-dependent conformational changes related to the closed↔open transition. Even then, the number of spectral couplings in a channel labeled with four nitroxide probes may preclude mechanistic interpretation, a problem that was overcome by using tandem constructs that allow for the introduction of only two spin probes into the channel. Thus, it was possible to conclude that the closed→open transition is associated with 10° rotations of the outer helix relative to the bilayer normal and plane, respectively, coupled to a 30° rotation relative to the helix axis. The narrow constriction at the cytoplasmic end of the path leading to the cavity in the KcsA structure increases in diameter by 2–3 Å. But, the range of the motion is such that it remains unclear whether the gate is at this constriction or whether the large-scale motion of the outer helix is coupled to more subtle changes in the selectivity filter that may contribute to the observed conductances changes.

To close the Symposium, R. MacKinnon (The Rockefeller University) gave the Keynote Lecture in which he summarized what has been learned from the KcsA channel structure (Fig. 1, left). Given the overall similarity between the sequence of KcsA and those of other potassium channels, the KcsA structure should serve as a template for understanding all potassium channels. Indeed, the KcsA structure has provided an immediate rationalization for many years of electrophysiological structure-function studies, in the sense that some features were predictable from the functional studies. The structure also revealed four important features that were not predictable from previous experiments: (1) that the pore-lining in the selectivity filter is formed by peptide backbone carbonyl oxygens from the signature Val-Gly-Tyr-Gly-Asp sequence; (2) that a dense network of Trp residues surrounds the pore-lining peptide strands and form hydrogen bonds with the Tyr in the middle of the signature sequence, which may provide rigidity to the pore structure; (3) that there is a large water-filled cavity, containing ~25 H_2O molecules, in the middle of the structure; and (4) that four α-helices, the pore helices (one in each subunit), are organized such that their COOH-terminal ends point toward the center of the cavity, thereby providing a negative elec-

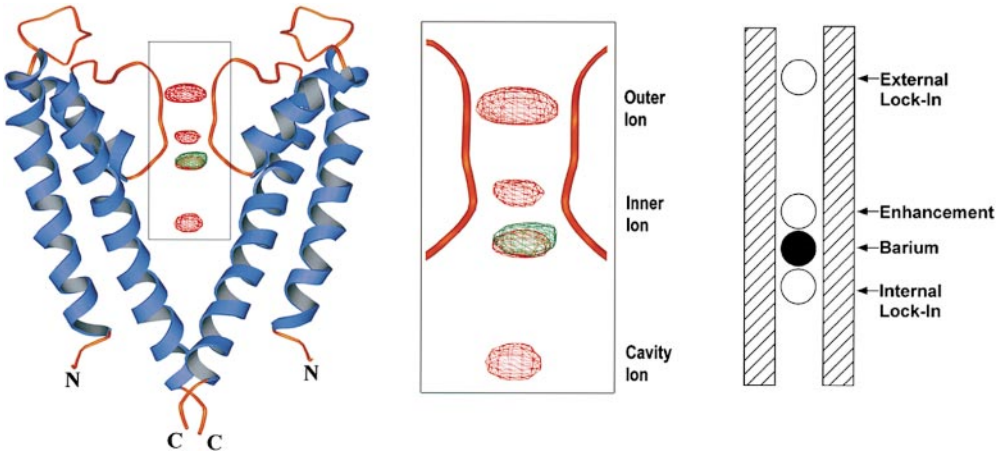

FIGURE 2. Different views of Ba^{2+} binding in potassium channels. (Left) Results from X-ray analysis of KcsA channels. The figure shows two of the four KcsA subunits and the superposition of electron densities due to Ba^{2+} binding (as green mesh) and Rb^{+} binding (as red mesh). (Center) The positions of the ion peaks at higher resolution. Ba^{2+} binds at the inner of the two inner Rb^{+} (or K^{+}) sites, and the Rb^{+} density in the cavity is comparable to the densities in the selectivity filter. (Right) The ion positions as deduced by Neyton and Miller (*J. Gen. Physiol.* 92:549 and 569, 1998). The external lock-in, enhancement and internal lock-in positions (○) denote the monovalent cation positions. Figure is reproduced from Y. Jian and R. MacKinnon (*J. Gen. Physiol.* 115:269, 2000).

trostatic potential that will promote ion occupancy in the cavity.

To date, the KcsA structure's major impact has been to improve our understanding of ion permeation. The fine details of ion movement through the pore remain to be worked out. But the structure constrains the movement to be in a single file in which a column of K^{+} and H_2O moves in "discrete" transitions between a small set of low energy configurations. Recent improvements in structure resolution to 2.7 Å provide evidence for three ions in the narrow selectivity filter, which illustrates how effectively backbone carbonyl oxygens can solvate the permeating cations and further reinforces the "long-pore" concept. The water-filled cavity in the center of the structure remains enigmatic, however. First, is the existence of the cavity just a consequence of protein packing demands and the need for the channel to undergo closed↔open transition, which requires that the space be filled with something else than protein? Second, why are ions in the cavity so localized that they show up in the crystal structure? Even though the pore helices would provide a negative potential in the cavity, it is not clear that the potential minimum should be as well-defined as it is. So, is the ion localization a consequence of the need to pack H_2O around the ion, which could serve as a mechanical splint that keeps the ion in place? Also, is the ion localization mechanistically important reflecting that the cavity serves to feed ions to the selectivity filter? The effective ion concentration in the cavity will be ~2.5 M; but, that is not the whole story because the pronounced localization of the ion in the cavity implies that the configurational entropy will be less than that for ions at a similar concentration in the bulk solution. Successful ion entry into a pore that is occupied by several cations, therefore, may depend on this highly organized arrangement. In any case, whereas the cavity is selective to monovalent rather than divalent cations, Ba^{2+} binding can be detected at the intracellular end of the selectivity filter with electron densities corresponding to two monovalent cations in the selectivity filter and one in the cavity. As can be seen in Fig. 2, this arrangement is remarkably consistent with the conclusion drawn by J. Neyton and C. Miller based on an analysis of the K^{+} dependence of Ba^{2+} block of Ca^{2+}-activated potassium channels.

The correspondence between the electrophysiological data of Neyton and Miller and the X-ray data of Jiang and MacKinnon (Fig. 2) highlights, again, an important theme of the Symposium, namely that the immediate impact of a new structure will be commensurate with the amount of preexisting data relating to that structure. The KcsA structure had an immediate impact on ion channel research because the structure provided a foundation for interpreting a large body of structure-function studies on many different types of potassium channels. A remarkable outcome of this re-

interpretation is, how well the tentative conclusions of the structure-function studies agree with predictions based on the KcsA structure. But, more importantly, the structure provided insights that never could have been obtained from the indirect approaches. Therefore, the importance of a structure is not so much the structure itself, however beautiful, but the insights it provides: first, by serving as a gold standard for interpreting previous experiments; and second, and most importantly, by serving as the framework for the design of new experiments to probe specific mechanisms or for calculating energy profiles for ion permeation or gating transitions, which would need to be tested experimentally. That is, the structure provides the anatomical underpinnings for achieving physiological insights, whereas function depends on the energetics (and dynamics) of the system of interest. The relationships among structure, energetics, and function are subtle and difficult to evaluate. Knowing a reference structure imposes an important, qualitative change on the way in which we think about molecular structure and function, and puts increasing demands on the experimental design and interpretation. But, the future is bright because we are moving out of the black box.

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