

# Membranes

## Membrane form and function in finer focus

### Editorial overview

### Martin Caffrey

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I am interested in the structure and function of membranes and membrane components. One area of current activity in the group concerns the crystallization of membrane proteins using lipidic mesophases. Another takes a rational design approach to lipid function in transport and controlled-release applications. Our laboratory hosts the Lipid Data Bank (<http://www.ldb.chemistry.ohio-state.edu>), a web-based relational database of lipid phase properties, miscibility and chemical structure.

#### Abbreviations

<b>BR</b>	bacteriorhodopsin
<b>DPPC</b>	dipalmitoyl PC
<b>HR</b>	halorhodopsin
<b>PC</b>	phosphatidylcholine
<b>PS</b>	phosphatidylserine
<b>PSI/II</b>	photosystem I/II
<b>SRII</b>	sensory rhodopsin II

When the invitation arrived to act as editor for the Membranes section of the August 2002 issue of *Current Opinion in Structural Biology*, I didn't hesitate to accept. It transpired that the remit of the assignment was perfectly in line with the objectives of a graduate level course I offer on alternate years to biochemists, biophysicists and chemists here at Ohio State University. The course deals with membrane protein structure and function, with an emphasis on structures published in the previous two to three year period. Each student is responsible for a single protein and is asked to investigate how the new structure informs function. Other issues addressed include the disparity, if any, between the new structure and earlier models, how the protein sits in the membrane and interacts with lipids and other proteins, and the research directions inspired by the latest structure. Given that the rate-limiting steps in structure determination of membrane proteins by crystallographic means include protein and crystal production, the students are expected to devote considerable effort to researching these aspects of their chosen system.

And so, taking on the editorship of the 2002 Membranes section was, in effect, killing two birds with one stone. I was at once provided the opportunity to have current highlights in the area of membrane structural biology reviewed by experts and to produce a document that will become an integral part of the curriculum of a graduate-level course addressing the latest word in membrane structure and function.

As editor, I was invited to assemble a collection of up to a dozen articles in the subject area, of which three or four might represent lipids. Accordingly, included under the rubric of lipids are contributions on experimental aspects of lung surfactant, and lipid domains and rafts, as well as a review of progress in the modeling of membrane lipids. The systems reviewed under the heading of proteins are the water and chloride channels, the calcium ATPase and the halorhodopsin (HR) chloride pumps, the sensory rhodopsin II (SRII) photo-receptor and photosystem II (PSII). The section also includes a review of a very promising new approach for crystallizing membrane proteins that employs antibodies. In what follows, I present a summary of each contribution, along with miscellaneous observations of my own.

#### The skinny on fat rafts

The review on rafts by Erwin London (pp 480–486) begins with an historical overview of this relatively new and rapidly evolving field. It introduces these

membrane subassemblies as domains of 'defined' lipid and protein composition in eukaryotic cells. The lipid component is enriched in saturated lipids, sphingolipids in particular, whereas the proteins are generally of the glycosylphosphatidylinositol (GPI)-anchored type bearing saturated lipid anchors. This compositional distinctiveness endows rafts with particular physical, chemical and biochemical properties. The discovery of rafts can be traced to early studies of lipid equilibrium phase behavior, to the isolation of detergent-insoluble membrane fractions (the much-used raft phenotype) from red blood cells and, subsequently, to membrane sorting and trafficking processes in cells. The realization that cholesterol can induce formation of what has come to be referred to as the lamellar liquid ordered phase and, by extension, of phase-separated domains was also important in the development of a model for raft genesis.

London limits his review to the use of fluorescence quenching as a tool for examining lipid domain structure and formation in bilayers that serve as models for biomembranes. The origins of the fluorescence quenching approach in such applications can be traced to Jerry Feigenson's laboratory at Cornell University, where studies of lipid and protein partitioning and association, and phase separation in membranes were initiated in the mid-1970s (see [1,2] for example). The method under consideration in the London review makes use of the ability of bromide- and nitroxide-labeled lipids to effect quenching when situated next to fluorescently tagged lipid molecules. It allows the sensitive detection of domains of distinct compositions in dispersed systems.

The method has been used to examine how cholesterol modulates the phase behavior of glycosphingolipids, sphingomyelin, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS), with implications for raft formation in the inner leaflet of the plasma membrane. A variation of the original method that exploits the thermal sensitivity of domains has been implemented in a survey of the ability of different sterols to form separate domains in combination with saturated lipids. Some work has been done with lipidated peptides with a view to establishing how partitioning into domains depends on the character of the acyl chain. Studies are in progress to examine the interaction of transmembrane helices with lipid domains.

Although fluorescence quenching has proved to be useful in studies of lipid domain formation and stability, the reviewer is careful to note its shortcomings. The method requires the use of lipids to which are appended potentially perturbing fluorescent or quenching moieties in the hope that they reliably relay information on the system under investigation and are free of artifacts. Unfortunately, the method has thus far been limited in its use to model membranes and has not found application in cellular systems. Furthermore, the method calls for high

quencher concentrations, which limits the mixture types that can be studied.

Other methods are mentioned briefly in the review, including fluorescence energy transfer, fluorescence microscopy, atomic force microscopy, calorimetry and diffraction. Most promising in this regard is the very recent study in which fluorescence microscopy was combined with resonance energy transfer between engineered fluorescent proteins to reveal, in a most direct way, raft formation in the inner leaflet of the plasma membrane of intact cells [3]. A host of questions that remain to be answered in this growing area have been identified in the review. The sense is that, although some of the basics regarding domain formation and stability are in hand, a considerable amount of work remains to be done before we come to appreciate fully how rafts contribute to the life of the cell.

### Breathing easy

Many organisms engage in gaseous ( $O_2$ ,  $CO_2$ ,  $H_2O$ ) exchange with their environment, primarily by way of the lungs. As an organ, the lungs provide a large surface area and a specialized interface for this vital to and fro. Lung surfactant creates that monomolecular interface and, despite considerable effort over the past half century, the molecular details of how the surfactant does what it does are not understood. However, important recent progress has been made, as reviewed by Stephen Hall and his associates (pp 487–494). The relevant issues are addressed in the context of two transitions. The first concerns the movement (adsorption) of material from multilayered lipid–protein (surfactant) vesicles in the aqueous phase on one side of the interface to the interfacial film itself. The adsorption process, which is quite rapid, is integral to the inflation of the collapsed or water-filled lung. The second transition has to do with (preventing) the collapse of the interfacial film during exhalation, when the lungs contract. Both processes are examined in the context of equilibrium and nonequilibrium lipid phase behavior.

On the basis of fluorescence recovery after photobleaching (FRAP) measurements, Hall and colleagues argue that it is the surfactant proteins (SP-B, SP-C), and not lipid fluidity, that have a major impact on adsorption. Furthermore, they make the case that interfacial boundaries of coexisting phases in the surfactant vesicles do not promote adsorption either. Rather, they propose the existence of a highly curved intermediate structure, a tether if you will, between the interfacial film and the vesicle. Although structures of the type that make up the lipidic inverted hexagonal phase have been proposed to serve this function, worth considering also are conduits with cubic symmetry, as in lipidic cubic phases and cubic membranes [4].

The original view (classical model) of the interfacial monolayer at surface pressures above that which trigger collapse under equilibrium conditions,  $\pi_c$ , is that of a

condensed, ‘solid’ film enriched in dipalmitoyl-PC (DPPC), one of the major surfactant lipids. But as DPPC represents less than half the phospholipids in lung surfactant, the classical model must account for its selective enrichment in the interfacial film. Hall and colleagues argue that this would require the preferential movement of DPPC from the surfactant vesicle to the monolayer and/or selective elimination of components other than DPPC from the monolayer. A convincing case is made that counters the classical model and argues in favor of a dynamic process that creates a metastable, kinetically trapped fluid monolayer with the properties of an amorphous film. Selective enrichment in DPPC is not called for in this proposal. The analogy to a supercooled liquid is made.

The structures that form upon collapse of the interface, and that must be avoided, are considered in the context of simple models as bilayer folds and buds. The relative rates of interface compression and collapse determine access to the proposed metastable amorphous state. It is clear therefore that determining what is formed, and how it is formed, upon collapse is another critical aspect of surfactant function yet to be resolved.

### Time to trash the test tubes?

Fast desktop workstations and modeling software are providing the means to simulate the lipid component of bilayers in atomic detail at time and length scales that are approaching those considered biologically relevant. Larry Scott (pp 495–502) reports recent progress in the area by first introducing the methodological advances in the field. One of these concerns simulation force-fields for describing polyunsaturated lipids of the type found in retinal tissue, for example, in which extraordinary flexibility has been shown to prevail along the entire acyl chain. Periodic boundary conditions have been introduced that allow the simulation of lipid redistribution across unsymmetrical bilayers — the hallmark of most biological membranes. Furthermore, considerable progress has been made in the treatment of electrostatic interactions in the low temperature lamellar phase. Simulations are now taking into consideration the surface tension at the lipid/water interface, which can be modeled as the sum of a large repulsive energy in the acyl chain region and an equally large attractive energy in the polar part. With bigger simulation cell sizes, an artificial applied surface tension ensemble would appear to be unnecessary. A new simulation approach has been introduced that enables a significant reduction in computational time by facilitating egress from local energy minima.

Recent simulation work on relatively simple glycerolipid/water systems has allowed the calculation of the rotational diffusion of chains, headgroups and entire molecules in ‘fluid’ lipid bilayers. A separate study provided insight into the hydrophobic and hydrophilic interactions of ethanol in PC bilayers. Another focused on the stepwise hydration of PC lamellae, for which the agreement with

diffraction measurements is particularly striking. The simulation is consistent with an immediate hydration shell consisting of 12 water molecules that forms about the lipid headgroup. A reduction in the surface area occupied by anionic PS compared to zwitterionic PC was accounted for by hydrogen bonding between the amino and phosphate groups of adjacent PS molecules in the bilayer plane. Other simulations call into question the use of monolayers to model bilayer behavior. Here, order was found to extend further down the acyl chain in monolayer-associated phospholipids at the air/water interface than in hydrated bilayer lipids. The successful simulation of a lipidic cubic phase was also realized during the period covered by the Scott review.

With a view to simulating more biologically relevant membranes, it is comforting to note that significant progress has been made in modeling cholesterol in hydrated lipid bilayers. This has revealed the well-documented condensing and chain ordering effects of cholesterol in fluid bilayers of saturated lipids that are consistent with experiment. The simulations also show that one face of the cholesterol molecule and the *sn*-1 chain of the glycerolipid dominate the interaction. Hydrogen bonding of the sterol hydroxyl moiety with the glycerolipid linkage and with water was observed. The simulations have been extended to include a number of cholesterol precursors and derivatives, with interesting insights as to their interactions in lipid membranes. Modeling of a DPPC/cholesterol system has provided evidence for the emergence of the lamellar liquid ordered phase at about 10 mole% sterol. This is a very significant result, despite the fact that the experimental value is closer to 20 mole%.

Up to the fairly recent past, theoretical and computational modeling has been limited to length and time scales several orders of magnitude less than those relevant to most biological processes. The thrust now is to extend these and some progress has been made with extrapolations to fractions of microseconds. It is with bated breath then that we await the development of simulations describing, in terms of atomic interactions, phase separation and domain formation in membranes that include both lipids and proteins.

### Locking down the lattice

The pioneering work of Michel and his colleagues [5] on the bacterial photosynthetic reaction centers demonstrated unequivocally that diffraction-quality crystals could be produced for use in high-resolution structure determination of integral membrane proteins. However, their surfactant-based, or *in surfo*, crystallization method has not proven to be the panacea, in that it does not appear to work with all membrane protein types. Indeed, the community was forced to wait for the introduction of a distinctly different crystallization approach, one that uses a lipidic mesophase of the cubic type [6], for a high-resolution view of the light-driven proton pump bacteriorhodopsin (BR) [7].

Interestingly, this is the same protein that Michel abandoned in his early attempts at generating diffraction-quality crystals of membrane proteins by the *in surfo* method. Carola Hunte and Hartmut Michel (pp 503–508) review recent successes with a new approach for crystallizing membrane proteins. It makes use of antibody fragments to effect, what amounts to, an orderly molecular soldering together of the unit cell components.

Most membrane proteins can be viewed as being amphipathic, having defined polar and apolar surfaces. Crystallizing such macromolecules by the *in surfo* method requires that the apolar band be cummerbunded by detergent and that the so-called type II crystal lattice be stabilized by polar contacts between proteins [8]. However, as noted, not all membrane proteins crystallize by the standard *in surfo* method. Failure has been ascribed to inherent protein flexibility and to conformational inhomogeneity. Also at fault is a relatively diminutive polar surface that is simply too small to extend beyond the surfactant swath and make molecular handshakes with neighboring proteins in the crystal. The new antibody fragment method makes good these deficits. The fragments can be tailored, to a degree, to fill the aforementioned gaps and to create stable protein–protein polar contacts within the crystal. Furthermore, by using high-affinity recombinant antibodies raised against a discontinuous epitope on the native protein surface, flexibility in the protein–antibody fragment co-crystal is minimized and conformational homogeneity is favored. All contribute to producing a well-diffracting crystal.

In their review, Hunte and Michel describe the three published structures that have benefited from this new crystallization approach. The cytochrome *c* oxidase and cytochrome  $bc_1$  complex structures originate from the authors' own laboratories, whereas that of the KcsA potassium channel is from Rod MacKinnon's group. The former made use of monoclonal *Fv* (~30 kDa) fragments, whereas the latter channel work was done with *Fab* (~50 kDa) fragments. The co-crystallization approach was either necessary to get crystals of diffraction quality or provided better crystals with enhanced resolution. The method relies on the use of homogenous, monovalent antibody fragments. Native antibodies, which are bivalent and inherently flexible, are not considered suitable.

In the cytochrome *c* oxidase and potassium channel co-crystals, antibody fragments created the sole crystal contacts. In both cases, the target protein and, presumably, associated detergent are locked into a continuous, tethering proteinaceous scaffold. Perhaps this is not unexpected because, in the case of the potassium channel at least, the co-crystals contain five times as much antibody as channel protein. The  $bc_1$  complex represents an interesting, and perhaps counter-intuitive, case in that it has an exposed polar domain twice as big as its apolar part. Still, *Fv* was required to produce crystals in which contacts were

mediated primarily by antibody. The latter work was extended impressively when the  $bc_1$  complex was crystallized in association with its cytochrome *c* substrate. The antibody fragments affected neither substrate binding nor complex activity.

Added features of the antibody approach have been identified in the review. These include fast and facile purification, given that the recombinant antibody can be procured with an affinity tag. Thus, in the case of cytochrome *c* oxidase, the detergent-solubilized, oxidase-containing membranes were mixed with periplasm in which the strep-tagged *Fv* had been expressed; the complex was isolated by single-step affinity chromatography. However, as noted by the authors, this method did not work with the  $bc_1$  complex under the conditions used. An additional feature of the antibody approach is highlighted in the potassium channel work. Here, the phases used in structure determination were obtained by the relatively straightforward method of molecular replacement. This tack benefited from the conserved nature of the antibody immunoglobulin fold and from the known crystal structure of a related *Fab* fragment.

Hunte and Michel caution that producing high-quality antibody fragments of the type used in crystallizing membrane proteins is a nontrivial undertaking. With a view to making the method more accessible, they emphasize the need for improvements in antibody screening procedures. Furthermore, they describe an *Escherichia coli* strain with an oxidizing cytosol for use as an expression system in which the yield of properly folded antibodies (with disulfides intact) far exceeds that of the more traditional extracellular, periplasmic expression system. Another approach for increasing the hydrophilic surface of a target membrane protein considered in the review involves covalent linkage to a soluble protein. Producing such a fusion protein is routine if the expression system is in hand. However, adding the soluble protein to one or the other end of the target may not work because of the additional flexible elements introduced that militate against crystallization. As a way around this, splicing between two transmembrane helices has been tried in the case of the lac permease. However, the approach, although not compromising permease activity, has not yet yielded a high-resolution 3D structure.

Of the six membrane proteins reviewed in this section, four were crystallized by means of the now standard *in surfo* protocol. The other two made use of the *in meso* method. The article by Hunte and Michel describes what amounts to a variation on the original *in surfo* method. Earlier this year, yet another approach was introduced, which begins by solubilizing the membrane protein (BR was used) in a discoidal lipid/detergent mixed micelle and effects crystal growth facilitated by a subsequent heat-induced phase transition [9]. This may represent a variation on the 'traditional' *in meso* method, given the gel-like

nature of the high temperature phase. With these assorted crystallization strategies, today's structural biologist has available a reasonably wide array of tools for producing diffraction-quality crystals of membrane proteins. The focus may now be turning to the last major roadblock en route to mapping the membrane protein structure landscape, that of protein production.

### Aquarius

The hydrophobic effect, which is key to the form and function of membranes, has its origins in the 'narcissistic' properties of water. Despite the 'affinity' that water has for itself, it must be partitioned within and without the cell. Indeed, maintenance of a proper cellular water balance is the hallmark of a healthy organism. Defects in water transport lead to a host of disease states. Integral to proper balance is the ability to regulate transmembrane water flux. A great variety of water channels have been identified that serve this function. Some are specific for water (aquaporins), whereas others handle water and glycerol (aquaglyceroporins). As with other channels reviewed in this section, the question that looms large with regard to function has to do with how their enormous flux characteristic ( $10^9$  water molecules per second) is reconciled with their exquisite selectivity. In the review by Yoshinori Fujiyoshi *et al.* (pp 509–515), we are told of the very significant recent progress in this area, which relies on structural insights provided by electron and X-ray crystallography. The authors also discuss how molecular dynamics simulations extend this structural information to the realm of transport kinetics, mechanism and selectivity.

The story of how the high-resolution structure of the water channel was elucidated is not unlike that for PSII, as recounted in this section by James Barber. In both cases, an atomic model was provided originally by electron crystallography. In the case of the (human) aquaporin, this was subsequently refined, confirmed and extended on the basis of X-ray crystal structure work performed on a bacterial aquaglyceroporin and a bovine aquaporin. Earlier electron crystallography and microscopy measurements had demonstrated that the channel exists as a tetramer, the monomers of which each contain a single pore. The pore itself is primarily hydrophobic, with the occasional polar moiety at strategic locations. It is formed at the confluence of six, highly tilted transmembrane helices, which take the form of a right-handed bundle, and a pair of short helices that cross the membrane from either side. The latter meet with their partial positive helix dipoles abutted at the mid-plane of the membrane. This calls to mind the helix dipoles employed by the KcsA potassium channel and the CLC chloride channels, referred to elsewhere in this section (see below). Each mini-helix of the water channel originates from a conserved asparagine–proline–alanine (NPA) sequence motif, where the two prolines stack at the bilayer center. The amido moieties of the NPA asparagines extend into the pore and hydrogen bond with passing water.

The pore has two prominent constrictions. The first and largest is 3 Å wide and is situated at the middle of the membrane, next to the NPA motif. Molecular dynamics simulations ascribe channel selectivity to this site. The narrowest region, which is only 2 Å wide, has a conspicuous cationic guanidinium moiety next to it and it is here that the asparagines of the NPA motif hydrogen bond with channel water. Simulation studies show that, in its passage across the channel, the water molecule undergoes a concerted rotation of 180° as a result of dipole coupling with the protein matrix. The mini-helices play a significant role here. For protonic conduction through the pore, a contiguous string of hydrogen-bonded water molecules is needed. This 'water wire' is essentially severed at the 2 Å constriction, which, in concert with the cationic nature of its immediate environment, serves to block proton passage. As pointed out in the review, a membrane that is 'tight' or nonleaky to protons is integral to life, which relies upon gradients for energy currency manufacture. The water channel appears to have been suitably crafted to function in this regard.

The work reviewed by Fujiyoshi *et al.* demonstrates convincingly the value of molecular dynamics simulations. Simulations extend the 'static' structural views provided by the complementary techniques of electron and X-ray crystallography into a world of action where mechanisms and the origins of selectivity can be explored, and experimentally testable hypotheses can be formulated.

### Hopping halides

The archaeal rhodopsins function in two distinct ways. The first is typified by SRII, introduced below, and serves as a photoreceptor. The second, represented by BR and by the chloride pump HR, operates as a solar-powered ion pump. BR and HR are remarkably similar proteins. They share up to 35% sequence identity, have the same covalently bound retinal chromophore and have a virtually identical membrane topology that takes the form of seven transmembrane helices. However, BR pumps protons out of the cell, whereas HR pumps chloride ions into the cell. Lars-Oliver Essen (pp 516–522) reports the 1.8 Å crystal structure of HR and uses it to argue that archaeal rhodopsins in general share a common pumping mechanism, despite their, as yet unexplained, distinct ion specificities and directionalities.

The crystals used to solve the structure of HR were grown from a lipidic cubic mesophase. They were found to include HR trimers very similar to BR, but with more extreme monomer tilting. Interestingly, the protein formed co-crystals with palmitate, a 16-carbon, saturated fatty acid. The lipid presumably was acquired as a nutrient from the medium used to culture *Halobacterium salinarum*, the organism from which the HR was obtained. Furthermore, it survived the isolation, purification and crystallization processes, which bring the protein into intimate contact with lipids and detergents. Separate biochemical evidence

suggests that palmitate plays a role in ion pumping. The fact that the crystal structure places the carboxylate of the fatty acid just 10 Å from the Schiff base and the bound chloride ion is not inconsistent with this proposal, as indicated by Essen.

In the case of BR, movement of  $\pi$ -helical segments in the transmembrane region of the protein accompanies photocycling. The lysine to which the chromophore is attached resides in the middle of one of these  $\pi$  segments in both BR and HR. It is argued that the photosignal is relayed to the transport site in both proteins by way of a restructuring of these  $\pi$  segments, which causes helices to tilt and the hydrogen-bonding pattern to change. In HR, a helical distortion consisting of a  $3_{10}$ -like sequence has been proposed to return to the  $\alpha$ -helical form as the chloride ion hops from the transport site to the release site in the protein. One other region implicated in pumping is the large interhelical loop that covers most of the extracellular surface of the protein.

In his review, Essen refers to biophysical studies that suggest the existence of three chloride-binding sites in the protein. The crystal structure reveals a chloride ion at what is called the transport site. This lies toward the middle of the protein, within 4 Å of the protonated Schiff base, and is close enough for electrostatic interaction. The crystal structure has been interpreted as showing a second, transient, halide-binding site toward the cytosolic side of the transport site. A pore on the intracellular side of this so-called release site is not evident in the structure, but presumably one forms during the photocycle to allow ion release into the cytosol. Evidence for a third halide site was not forthcoming from the X-ray-derived model.

The crystal structure is consistent with the following view of the events taking place during ion pumping. The guanidinium moiety of a critical arginine residue reorients to steer a chloride ion from the extracellular medium into the loading site next to the protonated Schiff base. Photoisomerization of the retinal causes the N–H dipole of the Schiff base to flip and to drag with it the chloride ion, depositing it in the release site. A pore opens to liberate the anion into the cytosol, the chromophore reverts thermally and the pump is restored to its original state ready for charging with another ion.

Given the similarities of BR and HR, it is not surprising that one can be engineered to behave like the other in terms of ion type pumped and photocycling. This suggests a shared mechanism and Essen discusses one that incorporates the so-called hydroxide hypothesis. In the case of BR, this involves the photoisomerization-induced movement of a hydroxide ion, created by proton abstraction from a water molecule hydrogen bonded to the Schiff base, from the extracellular to the cytosolic side of the chromophore. The proton is expelled extracellularly, whereas the hydroxide is reprotonated by the Schiff base

to form water. Thus, in both the BR and HR proteins, an anion crosses the chromophore: hydroxide in the case of BR and chloride in the case of HR. The suggestion is that this mechanism is common to all archaeal rhodopsin ion pumps. Presumably, specificity is imposed at this critical ‘chromophore-crossing’ step, as events leading up to and from it are relatively nondiscriminatory. The crystal structure introduced by Essen represents an important landmark en route to deciphering the molecular details of light-driven ion pumping in archaeal rhodopsins. Further progress in this area is likely to be rapid given that the ‘molecular movie’ of the BR photocycle has been made.

### Catching rays

Recent advances in the structural biology of photosynthesis are providing insights into the molecular workings of this ubiquitous process upon which much of life depends. The structure of PSI is available at atomic resolution. The recently published structure of PSII from cyanobacteria, as reviewed by James Barber (pp 523–530), although at less than atomic resolution, is serving to consolidate our views of how this part of the light reaction of photosynthesis comes about.

Water is the ultimate source of the electrons that are shuffled about during photosynthesis in many organisms. Effectively what happens in this solar-powered photolysis is that the electrons are driven through a series of carriers, which include PSI and PSII, and eventually are used to form reduced cofactors, such as nicotinamide adenine dinucleotide phosphate (NADPH). In this so-called light reaction, water is stripped of some of its electrons and converted to dioxygen ( $O_2$ ) and protons. The latter contribute, in part, to a proton gradient across the thylakoid membrane, the controlled relaxation of which drives ATP synthesis. NADPH and ATP are in turn used in the dark reactions of photosynthesis to fix  $CO_2$ , most immediately as a three-carbon acid.

The current view of how electrons flux through PSII is as follows. Light energizes electrons in bound chlorophyll *a* (P680), which move sequentially through bound pheophytin, plastoquinone A ( $Q_A$ ) and plastoquinone B ( $Q_B$ ). The latter accumulates two electrons, picks up two protons and then diffuses into the lipid bilayer to deliver its electronic cargo to PSI by way of cytochrome *b<sub>6</sub>f*. The P680\*+ so produced has a reduction potential sufficiently large to enable it to pluck an electron from a tyrosine residue in the D1 subunit ( $Y_2$ ) of PSII, which, in turn, is reduced by an electron derived from water. The latter is created by the so-called S-state cycle, which employs a manganese cluster ( $Mn$ )<sub>4</sub> to which water is initially bound.

The new crystal structure from the Saenger group, as reviewed by Barber, was obtained for PSII from a thermophilic bacteria at 3.8 Å, which is just shy of atomic resolution. However, it was sufficient to establish the positions in 3D space of transmembrane helices of the

reaction center and other protein subunits, most of the chromophores, the manganese cluster the nonhaem iron and several other extrinsic proteins. Tentative assignments were made for  $Q_A$ ,  $Y_Z$  and for its corresponding residue in the D2 subunit,  $Y_D$ . The crystal structure confirmed an earlier conclusion that P680 is not a special pair of excitonically coupled chlorophylls. Unfortunately, the resolution was not sufficiently high to identify amino acid sidechains or to definitively order helices. However, an attempt at this has been made by comparing the current model with those of the related bacterial reaction centers and PSI, both of which are available at atomic resolution.

Barber makes the important point that many of the structural details of how PSII is put together were already in hand and that the new crystal structure confirmed and extended these prior insights. The earlier structure models were based on a host of biochemical and biophysical studies of cyanobacterial and other reaction centers and photosystems, including those from higher plants. The most revealing of these employed electron crystallography and single-particle analysis. A case of 20:20 foresight!

Although the 3D crystal structure of PSII has been determined, Barber indicates that a vast amount of work remains to be done with a view to determining the structure and function of this multisubunit complex in its native thylakoid membrane. To begin with, higher resolution will be needed to identify the immediate protein environment of all chromophores and redox centers. With this, we can hope to chart the route taken by light-energized electrons that effectively originate in water molecules fastened to the manganese cluster on the luminal side of the complex all the way through to  $Q_B$  in its quinol form as it extricates itself from the grips of the D1 reaction center on the opposite stromal side. Higher resolution should help elucidate the roles of the multifarious low molecular weight subunits and of the peripheral cytochrome *c550*. The structural rearrangements that are needed for light-damaged D1 to be removed from the complex and to be replaced by pristine D1 are yet to be deciphered. The structure of larger supramolecular complexes, to include the light-harvesting complexes and other complexes important to PSII action, is also needed for a description of function in a native context. It seems likely that these goals will be realized by the combination of the tried and true methods that have brought us this far in our understanding of the light reactions of photosynthesis at a molecular level.

### Twin action

The CLC chloride channel is a ubiquitous protein, found in organisms from bacteria to man. Chloride channels function in transport and serve a role in muscle action. Mutations give rise to a number of genetic disorders. The crystal structures of two bacterial chloride channels determined by Rod MacKinnon's group, reviewed here by Raúl Estévez and Thomas Jentsch (pp 531–539), are

likely to prove useful in the rational design of drugs for the treatment of such disorders. The structures confirmed the double-barrel nature of the channel already envisioned on the basis of electrophysiological and biochemical studies. However, the crystal structure is at variance with biochemical membrane topology predictions. The severe tilting of the 17 intramembrane  $\alpha$  helices in each of the channel's two subunits and the fact that many of the helices cross the membrane only partially may explain the disparity. Cross-linking studies did not fare well either in that they led to a model in which a single pore emerged at the interface between the two channel monomers.

The chloride channel shares similarities with the KcsA potassium channel in that the ion-binding site is created by the termini of four intramembrane helices. In the case of the chloride channel, the bound anion is held, in what has been proposed to be the selectivity filter, by the partial positive charges associated with the electrical dipoles of the helices. In contrast, full charges are used by HR, the light-driven chloride pump. The intriguing point is raised that partial, as opposed to full, charges may account for the difference in the action of channels, which are fast, and pumps, which are slow. A consensus sequence (GXXXP) has been identified and proposed to be part of the selectivity filter, with conformational flexibility provided by the highly conserved glycine residue. A similar role is played by glycine in the selectivity filter of the potassium channel.

The crystal structure provides clues to the nature of the chloride-dependent fast gate, which controls individual pores. A conserved, negatively charged glutamate sidechain extends into the extracellular access site to the channel and may block it by electrostatic repulsion. However, the so-called common gate, which closes both pores, remains less well defined. Likely candidates for the common gate include the intersubunit contact region and a helix that extends from the cytosol into the chloride-binding site. The possibility that the fast and slow gates are coupled is considered in light of the fact that both depend on chloride ion concentration and on voltage.

Estevez and Jentsch indicate that future studies are likely to focus on identifying the gates and their mode of operation, and on the course of chloride ions through the pore. How the chloride channel interfaces with accessory subunits is another area of great interest and future work.

### Swimming against the (photon) tide

The sensory rhodopsin SRII is a light-sensing receptor protein in halophilic archaea responsible for what is called blue light avoidance. It is a retinal-containing, seven transmembrane helix protein that functions in phototaxis as part of a multiprotein complex. Light-induced structural changes in the protein are proposed to be transmitted by

side-to-side interhelical interactions with a neighboring transducer protein that, in turn, relays information to the motility apparatus by way of cytoplasmic phosphorylation. BR is a related microbial rhodopsin that functions as a light-driven proton pump. However, the spectral sensitivity of the two rhodopsins is quite different. Low-resolution electron microscopy failed to pick up structural differences between them that would account for this individuality. This led to the need for a high-resolution 3D structure to resolve the issue. John Spudich and Hartmut Luecke (pp 540–546) describe recent successes in this regard and the insights that the crystal structures provide about the function of the protein.

Crystallization of SRII was realized using the lipidic cubic phase method. Interestingly, archaeal lipids had to be included in the cubic phase matrix, suggesting that a more natural environment, with lipids familiar to the protein being crystallized, may be important in future applications of this crystallization method. Work along these lines is underway in this author's laboratory [10,11].

Comparing the crystal structures of SRII and BR provides important insights into the details of chromophore spectral tuning, whereby the  $\lambda_{\max}$  is 498 nm and 568 nm, respectively. The immediate retinylidene-binding pocket is very similar in both structures. Its polar, charged and strain-inducing character contributes to the spectral shift observed when the chromophore, free in solution with a  $\lambda_{\max}$  of 440 nm, is compared with the protein-bound form. However, careful examination of the two structures identified the positive end of a conserved arginine residue some 10 Å distant from the chromophore in SRII. The corresponding separation in BR is 9 Å. As pointed out by Spudich and Luecke, molecular orbital theory calculations support the contention that this long-range distance difference is the major factor contributing to the spectral individuality of the two systems.

A second important feature to emerge from the crystal structure is a tyrosine on helix G that sticks out conspicuously into what is likely to be the hydrophobic region of the supporting lipid bilayer. Convincingly, the authors argue that this conserved polar entity contacts the transmembrane region of the transducer molecule and that it serves a critical role in relaying the light trigger to the interior of the cell for the motility response.

The next most immediate challenge is to chart the structures of the SRII photocycle intermediates, as has been done successfully for BR. In parallel, the manner in which SRII couples to the cytoplasmic phosphorylation machinery, by way of the transducer protein, must be elucidated in atomic detail. The lipidic cubic phase, which comes complete with membrane and aqueous compartments, offers interesting prospects for the structure determination of such multisubunit complexes, some of which are membrane bound, whereas others are water soluble.

## Muscling metals

It was on the calcium pump that this author cut his biochemical teeth as a graduate student in Jerry Feigenson's laboratory at Cornell University in the late 1970s. I was interested in determining the optimum bilayer thickness for the activity of this transmembrane protein and in characterizing the nature of the interaction between the ATPase and its lipid environment [2]. And so, the protein was isolated from the sarcoplasmic reticulum of rabbit muscle, stripped of its native lipid and reconstituted into membranes of varying thicknesses prepared with synthetic lipids. Both the ATP hydrolysis and calcium transport activities of the reconstituted pump were monitored so that the coupling efficiency could be determined. At the time, very little information was available regarding the 3D structure of the protein. Amino acid composition was known and, from fluorescence quenching studies performed with nitroxide spin-labeled lipids, the view was that the bulk of the tryptophans were in the transmembrane portion of the protein.

And now, thanks to the efforts of Toyoshima *et al.* [12], we have the 3D structure of the calcium pump in atomic detail, which, as described in the review by Anthony Lee (pp 547–554), raises as many if not more questions than it answers. The structure, which is of the calcium-bound form, was obtained by way of a crystallization protocol that called for the addition of PC to stabilize the protein. It agrees well with much of the biochemical and biophysical data collected to date. Two calcium ions are buried between what have tentatively been identified as 'transmembrane' helices. Several possibilities for communication between the ATP-binding domain, the domain where phosphorylation occurs and the pathway through the protein traversed by calcium in the process of active transport are examined in Lee's review. Unfortunately, routes to and from the calcium sites on either side of the membrane are not obvious from the 3D structure in the way that they are for the potassium and chloride channel proteins.

Another question raised by the crystal structure has to do with how the protein sits in its native membrane. Tryptophans can sometimes point to the polar/apolar interface of a transmembrane protein and this clue is used to tentatively identify the location of the cytoplasmic surface of the membrane on the pump. Less clear, however, is the position of the luminal membrane surface, about which the tryptophan distribution on the protein is described as 'diffuse'. Two reasonable alternatives are considered and the possibility of hydrophobic mismatch playing a role in  $\text{Ca}^{2+}$ -ATPase function is proposed.

## Conclusions

Clearly, having the 3D structure of a membrane protein at atomic resolution is not enough to explain all aspects of its form and activity. This points to the need for additional biochemical and biophysical studies to establish the details of how the protein is situated and functions in its natural

membrane environment. Of course, crystal structures will serve as a basis for many hypotheses testable by the combined and separate uses of experiment and simulation. The past year or so has given us many new membrane protein structures, several of which are reviewed in this section. Others, which include monoamine oxidase B [13], the FecA iron transporter [14], the calcium-gated potassium channel [15], a lipid flippase (a homolog of the multidrug resistance ATP-binding cassette [ABC] transporters) [16] and an ABC transporter mediating vitamin B<sub>12</sub> uptake [17], will surely figure in future issues in this series. The pace at which new structures are appearing is quickening. With each round of new structures, membrane form and function comes into finer focus.

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