Perspective

Structure, Function, and Assembly of Membrane Proteins

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I HAVE RECENTLY HAD THE OPPORTUNITY TO CANVASS THE work and views of a group of scientists who attended a Nobel symposium on "Membrane proteins: Structure, function and assembly," held in Kalskoga, Sweden, in beautiful surroundings, partly in the last home of Alfred Nobel (*1*).

A major breakthrough took place when a three-dimensional crystal of the photosynthetic reaction center of *Rhodopseudomonas viridis* was analyzed at 3 angstrom resolution by H. Michel and collaborators (*1, 2*). Of the four protein subunits, the L and M polypeptides form the core, together with five different photosynthetic pigments. Both L and M consist of five returning α helices. The protein is inserted into the membrane as a solid block with no room for either phospholipids or much water. At the periplasmic side, the core interacts with a cytochrome. The amino terminus of the H subunit forms a membranous α helix, and the rest is a globular protein in the cytoplasm. The amino acid sequences derived from the genes contribute to a remarkably comprehensive picture of the atomic and topological structure of this protein. We can look forward to a model that will correlate the chemistry of the pigments that channel the electrons to conformational changes in the subunits. We have observed such changes induced by oxido-reduction in cytochrome oxidase (*3*), but we are lacking the three-dimensional information.

Khorana and his co-workers (*1*) combine biochemistry with molecular biology and site-directed mutagenesis. This is by no means a second-rate approach, as implied by modest Khorana, but rather a two-dimensional one with its admitted limitations. His experiments with denatured and even proteolytically cleaved bacteriorhodopsin, resurrected with phospholipids and retinal, have given him three parameters for the analysis by site-directed mutagenesis. Which amino acids influence protein folding, which retinal binding, and which proton pumping? Although specific amino acids have been identified as being required for retinal binding, thus far no decisive data have emerged to serve as clues to the mechanism of H^+ pumping. The approach is the best we have until suitable crystals of bacteriorhodopsin are available. Perhaps it is not surprising that the first crystals to yield diffraction data at high resolution were grown in a Max Planck Institute in Germany where research is independent of the weight of reprints and the grantsmanship of the applicant. A scientist in the United States who plans such a tedious, dull, and perhaps fruitless project is well advised to delete it from the grant application or hide it behind a screen of glittering experiments on site-directed mutagenesis. I hope that Howard Hughes Institutes will generate an atmosphere conducive to the growth of delicate crystals and other long-range projects such as the exploration of the underlying difficulties in crystal growth. Perhaps the difficulty in crystallization is the microheterogeneity of membrane proteins caused either genetically, posttranslationally, or by proteolytic or postdoctoral mutilations. Will it be possible to separate a homogeneous species either from native or denatured bacteriorhodopsin that is ready to unite into a well-ordered and symmetrical three-dimensional crystal?

Halorhodopsin, isolated by Oesterhelt and collaborators (*1, 4*), has about 50 percent homology with bacteriorhodopsin and common features such as the lysine, which binds retinal, the presence of seven helices, and a light-induced trans-to-cis isomerization of retinal during the photocycle. However, instead of a deprotonation of the chromophore, movement of chloride is visualized to take place. Thus, in spite of the charge difference between H^+ and Cl^-, the same basic mechanism can be formulated. The work of Oesterhelt, Khorana, and others has made it very unlikely that the bacterial rhodopsins operate via a water-filled channel. Therefore, we still need data that can be obtained by site-directed mutagenesis that will point to specific amino acid residues involved in the translocation of H^+ or Cl^-.

Nigel Unwin presented a model, based on cryo-electron microscopy and crystallographic methods, of the quaternary structure of gap junctions and the acetylcholine receptor. In the closed state, the subunits align approximately parallel to the axis of the channel. On removal of Ca^{2+}, the subunits of the gap junction protein tilt slightly, and an orifice appears at the surface. Thus we can easily visualize how such a small conformational change induced manually by Unwin in his physically constructed model could also be induced by Ca^{2+}, acetylcholine, or a change in the membrane potential in the natural membrane (*1*). To obtain clues to the mechanism of this induction we need to know more about the chemistry and specificity of the interaction between acetylcholine and the receptor, and we need to know how Ca^{2+} or a membrane potential influences the conformation of the subunits.

Rosenbusch presented data on crystalline porin (36,500 daltons), which is an unusual membrane protein composed of high polarity amino acids and with no extended hydrophobic stretches. According to several physical measurements including x-ray diffraction and infrared spectroscopy, it consists mainly of antiparallel β-pleated sheets (*1*). We do not know how *Escherichia coli* incorporates this protein into its membrane, but the fact that the pure protein can be reconstituted into artificial lipid bilayers suggests that we should think twice before proposing a single mechanism for protein insertion.

We can therefore now segregate two classes of membrane proteins involved in ion movements. One that includes the photosynthetic reaction center, bacterio- and halorhodopsin which are tightly packed with no room for phospholipids or a water channel. A second class that includes the acetylcholine receptor (AChR), the gated Na^+ channel, the gap junction, and porin contain a water channel.

Could a similar water channel be operative in an adenosine triphosphate (ATP)–driven ion pump such as the F_1F_0 proton pump of mitochondria, chloroplasts, and bacteria? In F_1F_0, a "channel" is formed by multiple copies of the proteolipid. We must assume that the directive for the closing and opening of this channel is transmitted to F_0 from the energy transformer in F_1 via several hundreds of amino acids. Which component of the electrochemical H^+ gradient serves as the signal? It appears from the work on Ca^{2+} adenosinetriphosphatase (ATPase) (*5*), on Na^+,K^+ ATPase (*6, 7*), and from the presentation by Ovchinnikov (*1*) that their active center of energy transformation may be as far removed from the lipid membrane as F_1 is from F_0 and that only about 20 percent of

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the protein is embedded in the membrane. Thus, in spite of the differences in the chemical mechanisms, common features between these pumps are emerging.

Studies of these ATP-driven ion transporters occupied a large part of the Nobel symposium. Great progress has been made in the elucidation of the F_1F_0 structure in the laboratories of Walker and Futai and other investigators. All the subunits of F_1F_0 from *E. coli* have been cloned and their sequences have been deduced (*1*). A topological model has been proposed that accommodates the participation of the eight subunits. Yet, once again, we are faced with the puzzle of the much more complex bovine heart F_1F_0, which has more cloned subunits with unknown function and more "coupling factors" (required for reconstitution) with unknown sequences. Recent data on the cooperative interaction between ATP and F_1 in the laboratories of Boyer, Penefsky, and Kozlov are consistent with the "binding change mechanism," which proposes that energy-induced changes in F_1 conformation result in changes in reactivity for the binding of inorganic phosphate (P_i) and adenosine diphosphate (ADP) and the release of ATP (*1*).

A few aspects of this area of research excite my personal vision, such as ion pump diseases. Mitochondrial myopathies have recently been studied by many investigators (*8*). It seems that cells of one patient described by Capaldi (*1*) cannot respire because of a deficit in ubiquinone–cytochrome c reductase in the skeletal muscle mitochondria, yet the cells survive, presumably by using glycolytic energy. The clinical success with vitamins K and C as a bypass, which greatly improved the exercise capacity of this patient, is paralleled by contributions to basic science emerging from studies of other patients with cytochrome oxidase diseases. Such studies may give us new insight into the role of subunits that are not required for the generation of an electrochemical proton gradient, yet are associated with the cytochrome oxidase complex. These polypeptides were given the status of subunits of cytochrome oxidase on the basis of "guilt by association." Now equipped with a set of antibodies against these polypeptides, Capaldi and others have obtained data that suggest that these subunits are tissue-specific and, in some tissues, are required for the assembly of an active complex (*1*). I believe that we are once again entering a phase of yesteryear when pathology helped us to understand physiology. What is pathology for one cell can be physiology for another. An uncoupler protein that is present in brown fat serves as an oven, allowing the survival of animals in the cold (*9*). Its presence in other cells would be fatal. Its triplicate structure with about 100 residues similar to that of two other mitochondrial proteins: the ADP-ATP and P_i transporters (carriers) was presented by Klingenberg (see *1*). I suggest that we should not call these proteins "carriers," a name that we should reserve for carriers such as valinomycin, nigericin, and fatty acids that actually move across the membrane.

The pathology of ATP-driven pumps, such as the Na^+,K^+ pump, reveals a defect in efficiency (the ratio of the Na^+ transported to the ATP hydrolyzed) in some tumor cells (*1, 10*). A dramatic change in this coupling ratio can be induced in the Ca^{2+} pump of sarcoplasmic reticulum by manipulation of the proportion of phosphatidylcholine and phosphatidylethanolamine (*11*). Once again, we must ask how the active site in the energy transformer senses changes in the membrane hundreds of amino acids away and how can we attack this problem of long-distance communication? Is Unwin's model, which seems quite plausible within the domain of the membrane, applicable to long-range signaling? Data on three-dimensional structures are needed, and physical chemists could help with model designs and experiments that directly demonstrate signal transduction over distances of hundreds of amino acids. Perhaps hemoglobin will serve as a model.

A revolution is taking place in the receptor field at the level of both structure and function. No unifying definition of receptors has been forthcoming. I shall not attempt it either, partly because I do not know how and partly because I have already done it (*12*). Actually, instead of defining, I have arbitrarily classified receptors into four major groups for the purpose of discussion. (i) The RGC receptors, which contain the receptor polypeptide (R), the guanosine triphosphate (GTP)–binding protein (G), and the catalyst (C); (ii) the water channels; (iii) the receptors for growth factors; and (iv) the transport receptors, such as those for low density lipoprotein (LDL) and transferrin. There are many receptors including drug, toxin, and chemotactic receptors that may or may not fit into one of these classes.

Numa has done impressive work on the AChR and the Na^+ transporter as well as the muscarinic receptor (probably an RG-channel receptor) by deriving the primary structure from clones of complementary DNA (cDNA) or genomic DNA. Single channel current measurements on hybrid AChR composed of *Torpedo* and bovine subunits produced in oocytes by expression of the corresponding cDNA's suggest a role of the ϵ subunit in the gating mechanism. Clues to differentiation were obtained by replacing the fetal γ subunit by the adult ϵ subunit, which induced changes in conductivity (*1*). It should be pointed out that Fujita *et al.* (*13*) have inserted by genetic manipulation the α subunit of the *Torpedo* AChR into the plasma membrane of yeast in the proper orientation. Having been converted to the faith of yeast molecular biology, I feel encouraged by this example of yeast thinking in the receptor field. On the other hand, as mentioned above, frog eggs have also become useful tools in studies of channels. Numa (*1*) showed that the Na^+ channel can function as a single large subunit even in the case of the rat brain protein, suggesting that the smaller associated polypeptides are either contaminants or regulatory subunits. Numa and collaborators have also cloned the muscarinic receptor and measured an atropine-sensitive inward Cl^- current after injection of the cDNA into oocytes (*1*). Hydropathy plots suggest seven transmembranous segments and similarity to β adrenergic and opsin receptors. This relationship is mirrored by the functional similarities and subunit interactions.

The successful reconstitution of RGC receptors in the laboratories of Gilman, Lefkowitz, Stryer, Ross, Sternweis, Cerione, and Helmreich (see *14*) have delineated the roles of R, G, and C. Stryer dealt elegantly with the RGC proteins in retinal rod cells and the succession of multiple amplification steps from a photon to cyclic guanosine monophosphate (GMP) resulting in an overall gain of over 500,000 (*15*). Yet another amplification takes place with the opening of a cation-specific channel in the plasma membrane by cyclic GMP (*16*). Many membranous functions appear to involve a member of the G family, which now includes, in addition to the mentioned receptors, the phosphoinositide (PI) cycle, ion channels, perhaps protein and sugar transport, not to forget cytoplasmic protein synthesis where it was first discovered. What are the relations between these G proteins and GTP-binding gene products of the ras oncogenes and the protooncogenes that have homology with the α subunit of G proteins? We have discovered two potential clues to such a relation. We have described several metabolic changes induced by these oncogenes which include a fourfold increase in glycolysis (*17*) and a fourfold decrease in sensitivity to PDGF (platelet-derived growth factor) as measured by the response of the PI cycle (*18*). We have proposed that, in analogy with the activation of yeast adenylate cyclase by ras p21 (*19*), a component of these pathways is influenced directly or indirectly by the oncogene product which either stimulates in the case of glycolysis or inhibits in the case of PDGF-dependent PI turnover. It seems likely that the future has surprises in store for us with the emerging control mechanisms of phosphorylation of receptors by protein kinase C,

cyclic adenosine monophosphate–dependent protein kinase, and some new highly specific protein kinases that are involved in coupling and decoupling of the receptor from the G protein. Antibodies and molecular probes are available for the analysis of the reactivity and distribution of the three subunits of G-binding protein. The pathological alterations induced by cholera and pertussis toxin have once again yielded clues to the function of G with its guanosine triphosphatase activity and its role in signal transduction. It is reasonable to predict that pathology in these complex pathways will be discovered and will give us better understanding of these and other G-protein diseases.

Modern receptor research has created a wave of excitement in the pharmaceutical industry. Old companies have opened new divisions of receptor technology, new companies are founded that are devoted mostly to receptors. Leading candidates are cardiovascular drugs, which last year were sold to pharmacists for more than $3 billion, and antianxiety drugs for over $0.7 billion. Drugs based on receptor binding studies appear on the horizon. Competition is fierce and drug delivery is a major obstacle. Since our most conservative companies are optimistic about the delivery of both drugs and profits we can look forward to probably important advances in the next decade.

What is the secret of protein traffic regulation, a key issue in the processes of endo- and exocytosis? The exciting advances in our knowledge of the structural requirements of presequences, discussed by Hurt, Blobel, and Wickner (*1, 20*), the role of positive charges, amphipathic helices, if indeed essential (*21*), and hydrophobic stretches, are the first steps to the next and elusive problem. There are specific receptors in the endoplasmic reticulum that allow the protein to enter, guided by the presequences. In spite of the many irreproducible successes and tantalizing failures with studies in mitochondria, evidence is slowly accumulating that receptors are present and will be conquered. I believe the receptors play a role in the accuracy of targeting, although it may be less exacting than DNA duplication. It seems to me that more information than is contained in the presequences is required for protein traffic between the numerous membranes and organelles in our cells.

I would urge a student who is interested in the structure and function of a membrane protein to learn the tools of biochemistry, biophysics, and molecular biology. When the function of the protein is not known, yeast or another cell that allows eviction of a gene is a good choice. Oocytes are now used successfully for the probing of structure-function relationships. The primary structure of the protein should be obtained by cloning and by determination of the amino acid sequence after purifying the protein using reconstitution as an assay. Relying on DNA or protein sequencing alone has led to mistakes. Clues to the mode of action can be obtained by site-directed mutagenesis, and any free time on Saturdays and holidays should be devoted to crystallization. For the study of membrane diseases antibodies should be prepared against each of the subunits, and a search should be made for the right patients. If the student is interested in the mode of action of oncogenes, a study of cells transfected with single oncogenes is probably the best model system; a search for changes in phosphorylation and dephosphorylation and for alteration in receptor profiles and properties and of the cytoskeleton is likely to be rewarding. Or, young students may even become interested in bioenergetics, in energy budgets, and control of efficiency, which are much neglected and exciting areas. If none of these approaches are appealing, another consultant might provide a more visionary list including chemotactic proteins and memory.

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