There is No Overkill in Biochemistry

Har Gobind Khorana, a Pioneer in Membrane Biology

Sadashiva Karnik and Sriram Subramaniam

Har Gobind Khorana pioneered study of transmembrane proteins at highest resolution possible through the application of biochemical techniques. His research on bacteriorhodopsin, a retinal based light driven proton pump from *Halobacterium halobium* spanned a range of ground breaking studies from membrane reconstitution, complete denaturation and refolding of a polytopic membrane protein, gene synthesis combined with genetically-directed protein modification and protein dynamic/kinetic analysis. His courage for tackling tough biochemical problems through enormous enthusiasm, building of a multi disciplinary team and maintain focus is a lesson for generations to come.

(left) Sadashiva Karnik obtained his PhD from Department of MCB, IISc and did post-doctoral work in the laboratory of Gobind Khorana at MIT. He joined Cleveland Clinic Lerner Research Institute, Cleveland, USA in 1990 and currently is a Professor of Molecular Medicine. His research focus is on peptide hormone G protein- coupled receptors.

(right) Sriram Subramaniam obtained his BTech from IIT Kanpur, India and PhD in physical chemistry from Stanford University. He carried out postdoctoral research in the laboratory of Khorana at MIT. He joined the faculty of Johns Hopkins University School of Medicine in 1992 and is currently Chief of the Laboratory of Cell Biology at National Cancer Institute, Bethesda, USA.

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Har Gobind Khorana (called Gobind with affection) exhorted members of his team to exercise the highest stringency in the care they took as experiments were carried out in the laboratory. "There is no overkill in biochemistry" was one of the constant refrains while he mentored young scientists. With characteristic humility and after two decades of pioneering contributions to the field, Gobind said in a lecture, "currently, we seem to be entering the golden age of integral membrane proteins". He foresaw the impact this 'golden age' would have on molecular biology of biomembranes and signal transduction. Two decades of his own research on two light-transducing integral membrane receptor proteins, bacterorhodopsin and bovine rhodopsin, created a decisive impact on this field and paved the way to the beginnings of this 'golden age'. Gobind's early research focused on the chemistry and biochemistry of nucleotides and polynucleotides (see accompanying articles) at the British Columbia Research Council, University of British Columbia at Vancouver, Canada (1952–1960) and Institute for Enzyme Research of the University of

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Wisconsin, Madison, USA (1960-1970). While in the final stages of completing the landmark projects on synthesis of tRNA genes and establishing the functionality of a synthetic tRNA gene at Massachusetts Institute of Technology, Cambridge, USA, Gobind made a radical change in his research direction by switching to decipher the 'codes' of membrane protein structure and function. In choosing this research problem Gobind was inspired by Efraim Racker during a sabbatical stint at Cornell University [1].

1. Changing Interest from Nucleic Acids Research to Biomembranes

With the goal of pursuing molecular neurobiology and signal transduction paradigms, Gobind initially explored simple membrane protein models such as the *E. coli* lipopolysaccharides and phospholipids. His efforts were focused on interactions of integral membrane proteins with phospholipids and reconstitution of membrane protein functions in synthetic lipid membranes. These pilot studies, which began in 1970, included cytochromes, sarcoplasmic ATPases, glycophorin, and the Ca-ATPase, and lasted nearly five years. During this time, Gobind formulated a clear commitment to study bacteriorhodopsin as a model system for understanding how membrane proteins work.

Mitchell's chemiosmotic hypothesis [2] that living cells harness chemical energy from a proton gradient generated across the biological membrane and Stoeckenius's discovery [3] that bacteriorhodopsin residing in the purple membrane of *Halobacterium* is a light-driven proton pump, which sustains this bacterium's life in high-salt milieu (4M NaCl) were backdrops for Gobind's choice of bacteriorhodopsin for his studies betw een 1975 and 1992. Now generally accepted as one of the great ideas of twentieth century biology, the chemiosmotic theory proposed that the proton gradient is generated across membranes to drive vital functions of a cell through synthesis of high-energy phosphate bonds (ATP), ion gradients, osmosis and more. How external sources of light or nutrient energy are coupled to generation of proton gradient in a cell was a mystery in 1975. In this context, the

choice of bacteriorhodopsin as a simple model system was sound for many reasons.

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The Start of Bacteriorhodopsin Biochemistry at MIT

From the beginning it was clear that bacteriorhodopsin is the only protein in the purple membrane necessary and sufficient for generating the light-driven proton gradient. Bacteriorhodopsin forms a crystalline lattice in the purple membrane. A low-resolution electron crystallographic structure of the bacteriorhodopsin trimer in the purple membrane was established in 1975 by Nigel Unwin and Richard Henderson at the MRC Laboratory of Molecular Biology in Cambridge, UK [4]. This structure indicated that the transmembrane portion of bacteriorhodopsin was composed of a bundle of seven helices. All-*trans*-retinal, bound covalently to the polypetide was shown as the chromophore in bacteriorhodopsin, making the protein the nearest relative of mammalian visual opsins which use an 11-*cis*-retinal chromophore (see the accompanying article on rhodopsin by Farrens and Sakmar). Like the visual opsins, bacteriorhodopsin displays light-induced spectral changes called the 'photocycle'. However, unlike the visual opsins, the cycle of reactions that follow light absorption in bacteriorhodopsin return the pigment to its initial state without dissociation of chromophore (retinal), ready for a new round of light-driven proton transport. While the biophysical aspects of bacteriorhodopsin in the purple membrane were gradually becoming better understood by optical and vibrational spectroscopic studies, a huge gap remained in connecting these structural and biophysical studies to the actual function of the protein in the membrane. This was the challenge Gobind recognized and began to tackle in his inimitable, systematic and thorough way, eventually culminating in the publication of over 100 papers on the subject, reflecting the work of almost as many colleagues and researchers who trained in his lab.

Gobind's team first developed new approaches to sequencing extremely hydrophobic transmembrane regions of bacteriorhodopsin and established the polypeptide sequence. In parallel, the

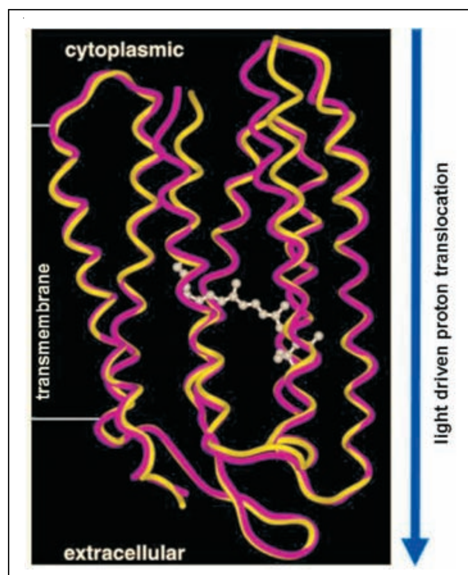

Figure 1. Open (pink) and closed (yellow) states of bacteriorhodopsin at 3.2 Å resolution derived by cryo-electron microscopy [6].

gene for bacteriorhodopsin was cloned. Sequencing the gene confirmed the polypeptide sequencing results and in addition revealed a highly unusual signal peptide sequence that was not present in native bacteriorhodopsin isolated from intact cells. Following this feat, two important features of the protein were quickly established. First, the experiments demonstrated that the N-terminus of the protein was located outside the cell membrane and the C-terminus was located inside the cell (see *Figure* 1). Second, all-*trans*-retinal was shown to be attached to Lysine-216 in the seventh helix in the middle of the membrane bilayer to form the chromophore for light absorption (*Figure* 2). Together, these discoveries quickly established a secondary structure model of bacteriorhodopsin. Soon afterwards, the Khorana lab showed that the native lipids surrounding native bacteriorhodopsin could be exchanged, without loss of function, with defined synthetic lipid membranes and vesicles. This observation led to the important discovery that the lipid composition was not central to the function of proton pumping. Proteolytic fragments of the bacteriorhodopsin were used to discover that the entire transmembrane helical bundle of bacteriorhodopsin was necessary for the light-driven proton pumping. The monumental achievement

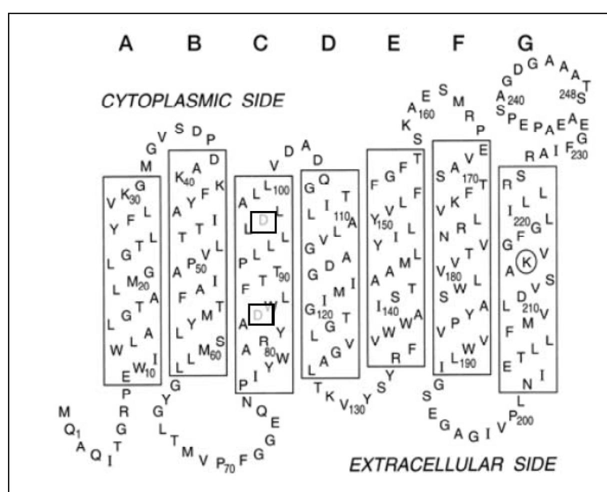

Figure 2. Secondary structure model of BR with Asp 85 and Asp 96 shown in squares and Lys216 shown in circle.

of this phase of the project was refolding of bacteriorhodopsin from completely denatured polypeptide by systematic addition of phospholipids, detergents and the chromophore while the denaturants were removed from the mixtures. This was thought to be impossible for membrane proteins until demonstrated successfully by Gobind's research team. These were seminal results that would have profound significance for later work with bacteriorhodopsin (see below). Additionally, it prepared the field of integral membrane receptor and channels for the advancements it witnessed a decade later.

The in-depth analysis of the membrane biology of bacteriorhodopsin allowed clear formulation of the key future directions and the approaches needed to accomplish these objectives. Gobind realized that chemical modification approaches traditionally used to define protein function would be both difficult and limiting to the scope of studies. Fortunately, strides made in the site-directed mutagenesis technology had already begun to yield insights in the study of enzymes. However, application of this technology to dissect the structure–function mechanism of bacteriorhodopsin posed formidable challenges. Methods for transformation of native *Halobacterium halobium* system had not been developed, but introduction of the bacteriorhodopsin gene in *E. coli* cells did not result in measurable protein production. Even when bacteriorhodopsin was expressed as a fusion protein with an *E. coli* membrane protein, the expression level was disappointingly low and the bacteriorhodopsin portion of the fusion protein did not fold to yield a light-sensitive pigment. Further, manipulation of the bacteriorhodopsin gene in *E. coli* posed additional technical problems because of the high GC content of the gene (~75%).

Gobind's team overcame these challenges through a series of innovations [5]. Returning to the gene synthesis technology invented in the early seventies by Gobind, they engineered a gene to express the 248 residue long bacteriorhodopsin polypeptide without any additional amino acids. The designed gene contained codons optimized for *E. coli* with unique restriction enzyme sites placed every fifty nucleotides throughout the gene to facilitate

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mutagenesis of any part of the gene. This synthetic gene produced misfolded bacteriorhodopsin at very high levels. Once this was accomplished, a two-step procedure was developed to isolate and purify the misfolded bacteriorhodopsin from *E. coli*. The purified misfolded bacteriorhodopsin polypeptide was first fully denatured and then refolded to functional bacteriorhodopsin by the methodology that was previously developed in the lab to establish denaturation and renaturation of native bacteriorhodopsin. These efforts took nearly four years of tireless work from a very talented group of individuals with great perseverance. During this period, no papers were published on this project. When the work had finally reached a stage that he was satisfied with, Gobind published five back-to-back papers in 1987 in the *Journal of Biological Chemistry* that provided a complete blueprint for structure–function studies of bacteriorhodopsin with a level of depth and breadth that stunned everyone in the field of membrane protein biology. The lessons learnt provided a solid foundation for structure–function studies of integral membrane proteins in general.

Beyond Deductive Analysis, Model for Photon-driven-Proton Translocation

Gobind was particularly inspired by an idea that was originally formulated by Manfred Eigen that proton conduction in proteins such as bacteriorhodopsin would occur much in the way protons were conducted in crystalline ice by a network of hydrogen bonds. Gobind felt that if every residue in bacteriorhodopsin capable of forming a hydrogen bond could be replaced one at a time by a similar residue not capable of forming a hydrogen bond, it would then be possible to define a path for the proton across the membrane. Simple as this idea was, implementing it was a heroic and monumental exercise. Over a five-year period, every aspartic acid, glutamic acid, serine, threonine, tyrosine, lysine and arginine in bacteriorhodopsin was replaced one at a time by residues such as alanine, asparagine or glutamine. Each of these mutant proteins was expressed, purified on a large scale and studied to determine the effect of the mutation on proton transport activity. Initial studies of their effects on function were carried out by

proton pumping assays in the lab, using a replica of the apparatus in Efraim Racker's laboratory. The scope of these studies was greatly enhanced with highly productive and strategic partnerships Gobind formed with physical chemists such as Kenneth Rothschild at Boston University and Maarten Heyn at the Freie Universitat in Berlin, Germany. These studies on the biophysical analysis of a very large number of site-specific mutants laid the intellectual foundation for a completely different way of thinking about the mechanism of proton transport than how it was envisioned by Manfred Eigen. Two key residues, Asp 85 and Asp 96 were identified as being critical for the function of proton pumping (*Figure* 2). Integration of these findings with the three-dimensional structure of bacteriorhodopsin, which emerged at about the same time, led to consolidation of the elegant discovery that these aspartic acid residues were positioned on either side of the protonated Schiff base to serve as an acceptor and donor in transit of the proton across the centrally located Schiff base [6].

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Gobind belongs to a small group of human beings who achieve greatness simply because they are thorough, perseverant and do not stop until all questions are answered to their satisfaction. A telling anecdote of this remarkable sincerity was a conversation with lab members in the mid-1990s regarding the future of bacteriorhodopsin research. As the work in his lab was gradually shifting away from bacteriorhodopsin, someone noted that it was a good time to move on from bacteriorhodopsin because we now understood how it worked. Gobind immediately stopped the conversation with the admonition that the problem of how bacteriorhodopsin worked was just beginning to be explored, and that it would continue to be worked on by people competent to make genuine contributions. It was clear that he had made the choice to move on from bacteriorhodopsin not because it was any less interesting to him than it was twenty years ago, but simply because he felt that a new set of tools were now needed to generate the next level of incisive insights into the mechanism of light-driven proton transport. He was thoughtful professionally and personally, and an extraordinary human being. His presence

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in the biochemistry field laid the foundation for the structural biology of membrane proteins, and illuminated the path for those who have followed his trail-blazing studies with bacteriorhodopsin. He trained a large number of exceptionally bright, dedicated and hard working scientists who have gone on to leadership positions in prominent institutions all over the world. His accomplishments and scientific legacy will continue to serve as an inspiration for many more future generations of scientists.

Suggested Reading

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