Understanding phospholipid function: Why are there so many lipids?

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In the 1970s, phospholipids were still considered mere building blocks of the membrane lipid bilayer, but the subsequent realization that phospholipids could also serve as second messengers brought new interest to the field. My own passion for the unique amphipathic properties of lipids led me to seek other, non-signaling functions for phospholipids, particularly in their interactions with membrane proteins. This seemed to be the last frontier in protein chemistry and enzymology to be conquered. I was fortunate to find my way to Eugene Kennedy’s laboratory, where both membrane proteins and phospholipids were the focus of study, thus providing a jumping-off point for advancing our understanding of phospholipids, particularly in their roles for non-signaling functions. After purifying and characterizing enzymes of phospholipid biosynthesis, membrane protein biosynthesis, phospholipid and membrane protein trafficking, and the cellular roles of phospholipids. After purifying and characterizing enzymes of phospholipid biosynthesis in Escherichia coli and cloning of several of the genes encoding these enzymes in E. coli and Saccharomyces cerevisiae, I was in a position to alter phospholipid composition in a systematic manner during the cell cycle in these microorganisms. My group was able to establish, contrary to common assumption (derived from the fact that membrane proteins retain activity in detergent extracts) that phospholipid environment is a strong determinant factor in the function of membrane proteins. We showed that molecular genetic alterations in membrane lipid composition result in many phenotypes, and uncovered direct lipid-protein interactions that govern dynamic structural and functional properties of membrane proteins. Here I present my personal “reflections” on how our understanding of phospholipid function has evolved.

In the beginning

My contributions to the biological sciences have been dependent on my early mentors, my scientific colleagues (students, postdocs, and established investigators), being at the right place at the right time, opportunity evolution of methods and techniques, and some scientific insight along with a good amount of luck. In 1960, I was fortunate to be admitted to Princeton University after attending a Detroit suburban high school. After initially majoring in mathematics, I switched to chemistry. I was fascinated with organic chemistry, which resulted in my lifelong interest in the chemistry of living systems. Taking physical chemistry and later physical organic chemistry from Walter Kauzmann I was in a position to alter phospholipid composition in a systematic manner during the cell cycle in these microorganisms. My group was able to establish, contrary to common assumption (derived from the fact that membrane proteins retain activity in detergent extracts) that phospholipid environment is a strong determinant factor in the function of membrane proteins. We showed that molecular genetic alterations in membrane lipid composition result in many phenotypes, and uncovered direct lipid-protein interactions that govern dynamic structural and functional properties of membrane proteins. Here I present my personal “reflections” on how our understanding of phospholipid function has evolved.

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obvious. After graduation and our marriage in June of 1964, we moved to Berkeley so I could begin studies at the University of California. The stipend was $2800 a year, a far cry from the $19,000 Ford had offered. Fortunately, Jerilyn worked as a nurse during most of our years at Berkeley, making our time there more comfortable. These were of course the wild years of the Vietnam War, the free speech movement, and the Reagan governorship. This was the polar opposite of the social atmosphere of the Princeton campus. The military service draft constantly made our future uncertain as fellow students, especially those with an M.D., disappeared on a regular basis. Most of us confined our time to the lab but thoroughly enjoyed the colorful events that surrounded us.

My first-year courses were in biochemistry (taught by Esmond Snell), a genetics course, and an intensive lab course where we spent all of our free time learning biochemical techniques. The genetics course relied heavily on what was known about the lac operon in the mid-1960s. This would be a very important exposure for my future use of lactose permease (LacY) as a model membrane protein. We also took seminar-based courses where each of us presented on a topic of our choice. I ran across a series of books in the biochemistry library that dealt with membranes. At the time, there were still arguments concerning the basic structure of membranes. A lipid bilayer as the core of membranes was proposed in 1925 (3), but arguments were still being made for proteins as the organizing framework for membranes. How proteins were organized in the membrane was unknown. A particularly interesting section of this book series proposed the organization of proteins into domains within a lipid bilayer. This was well before Singer-Nicolson proposed the “fluid mosaic model” (4) for membrane structure. Naively, I chose this as my seminar topic. The result was a near failure in the course due to my inability to recognize such “poorly documented scientific studies.” This was a lesson in the difficulties of challenging current dogma, a practice which I have tried to continue to this date. Interestingly, my research group later contributed to the body of evidence supporting lipid domains with specifically associated proteins in bacterial membranes (reviewed in Ref. 5). I chose to do my Ph.D. thesis under the direction of Esmond Snell because of the opportunity to learn methods of protein purification, protein characterization, and enzymology. Esmond was of course world-renowned for his work on enzymes containing pyridoxal phosphate as a cofactor. Esmond’s lab was heavy on postdoctoral fellows each working on their own enzyme. The presence of experienced postdocs was particularly important during Esmond’s one-year sabbatical, and his absence forced me to become independent and resourceful. I gained access to Dan Kosland’s newly arrived Beckman amino acid analyzer and Howard Schachman’s ultracentrifuge in order to determine the amino acid composition and molecular weight, respectively, of D-serine dehydratase (6). After devising a method for producing the apoenzyme followed by reconstitution, I used Esmond’s collection of B6 analogues to establish the structural features of pyridoxal necessity for enzyme binding and catalytic activity (7). While my project progressed smoothly, the outside world interfered at times. For example, free speech “terrorists” of the time were blowing up establishments of capitalism such as Bank of America branches, and on one occasion, a major electrical tower of Pacific Gas and Electric; the latter event shut down one of my ultracentrifuge runs. I filed my Ph.D. thesis at the Sproul Hall administration building while it was surrounded with state troopers and student demonstrators.

While I was in the lab, Dixon Riley discovered a new prothetic group residing in the histidine decarboxylase of Lactobacillus 30a (8). An N-terminal pyruvate residue is created by an autocatalyzed serinolysis of the peptide bond N-terminal to a serine residue in the proenzyme. Awareness of this discovery would be important to my later studies on phosphatidylycerine (PS)2 decarboxylase from Escherichia coli (9, 10).

My Ph.D. thesis did not produce any seminal results or earth-shaking principles. However, the training in the scientific method and rigorous molecular description of results along with a solid background in protein chemistry and enzymology would serve me well in the years to come. I owe a great debt to Esmond and the faculty at Berkeley for the opportunity to do research in their midst.

Making the move to membranes

The earlier encounter with the area of membrane structure in my seminar course, although traumatic, still piqued my interest, and I was eager to learn more. In the late 1960s, few integral membrane proteins had been purified in an active form. A search of the literature turned up the classic Fred Fox and Eugene (Gene) Kennedy paper (11) describing the identification of the membrane-associated M protein, which is the lacY gene product LacY. Gene’s group correctly suggested that the M protein carried out both energy-independent equilibration of substrate across the cell membrane as well as energy-dependent accumulation of substrate without modification of the substrate (12). This was in stark contrast to the phosphotransferase-dependent systems studied by Saul Roseman (13), which used metabolic energy to modify the substrate, resulting in accumulation. These differences in mechanism provided colorful debates at Gordon Research Conferences for several years between Saul, Gene, and H. Ronald Kaback as to the mechanism by which LacY transports substrate. Although Ron and Gene had differences in details, they agreed that a high-energy covalent intermediate was not involved in the LacY mechanism. Ron eventually established that an electrochemical proton gradient was the driving force for lactose accumulation, which was the culmination of what Ron termed the “Chemiosmotic Wars.” Ron of course went on to establish the detailed molecular structure and mechanism of LacY (14, 15). It was during these Gordon Conferences debates that I got to know Ron (Fig. 1), which resulted in a long and productive scientific friendship.

After hearing about LacY for so many years, I decided to bring my acquired expertise in protein purification and charac-

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2 The abbreviations used are: PS, phosphatidylycerine; PG, phosphatidylglycerol; PGP, phosphatidylglycerol-P; PE, phosphatidylethanolamine; CL, cardiolipin; PC, phosphatidylcholine; PCTP, phosphatidylcholine transfer protein; PI, phosphatidylinositol; PITP, phosphatidylinositol transfer protein; NAO, 10-N-nonyl acridine orange.
terization to bear on this protein, and thus make my entry into
the nascent field of membrane proteins. I arrived with my wife
and son David for a postdoctoral position at Harvard Medical
School in the late spring of 1969. Gene (Fig. 2) had of course
delineated most of the metabolic pathways for phospholipid
biosynthesis in mammalian cells and *E. coli* (summarized in
Ref. 16). His foray into membrane transport processes was a
new direction for the lab, as were attempts to purify membrane-
associated proteins. Over the next five years, the Kennedy lab
(Fig. 2) filled with what would become the future leaders of lipid
metabolism and membrane biology. Edward Dennis would
develop the important surface dilution model to explain the
kinetics of integral and peripheral membrane proteins that
used membrane-associated lipid substrates (17). He would
become a leading figure in phospholipase A₂ generation of lipid
second messengers. David Nelson would assume the lead
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authorship of Lehninger’s classic biochemistry textbook *Principles of Biochemistry*. William Wickner would join Arthur Kornberg’s lab after medical school, and accomplish the difficult purification of the *E. coli* DNA polymerase III (18). He would return to membrane studies to be a leading figure in the biosynthesis and assembly of membrane proteins. Christian Raetz (Fig. 2) would be Harvard’s first M.D./Ph.D. graduate. He would combine classical and modern molecular genetics with rigorous mechanistic biochemistry to map mutants in *E. coli* phospholipid metabolism and delineate the complex pathways for the synthesis of lipid A (19), the core membrane component of lipopolysaccharide present in Gram-negative bacteria. Carlos Hirschberg would go on to make seminal contributions in glycoprotein biosynthesis. Shortly after my departure, James Rothman joined the laboratory. He would receive the Nobel Prize in physiology in 2013, along with Randy Schekman and Thomas C. Südhof, for seminal work on intracellular protein trafficking. Dennis Voelker would begin to unravel the complex process of inter-organelle movement of phospholipids (20) and the role of phosphatidylglycerol (PG) as a lung surfactant antimicrobial agent (21). Continual interaction with all of these scientists over the years had a profound influence on my work.

I set out to purify the M protein (i.e. LacY). The assay was quite crude and based on the original specific radiolabeling devised by Fred and Gene (11) rather than reconstitution of transport function in proteoliposomes as later developed by Ron (22). I tried all of the available mild non-denaturing detergents available at the time with no success. Either the protein was not extracted, or if extracted, it was hopelessly aggregated. My scientific disappointments with the purification of LacY were compounded in the summer of 1969 with my mother’s diagnosis with terminal pancreatic cancer and the realization that my father had advanced Alzheimer’s disease. With the support of Gene and the American Cancer Society (which extended my two-year fellow to three years), I took a nine-month leave from science. My parents both passed away in December of 1969, and after settling their affairs, I returned to the lab in the spring of 1970 to continue with the LacY purification. However, after several months, it became obvious that this project was a failure. Eventual success in purification of LacY would depend on the availability of octyl glucoside and dodecyl maltoside and the efforts of Hastings Wilson and Ron Kaback 10 years later (23). However, I would return to LacY some 20 years later as the model protein in my studies of lipid-protein interactions.

During my absence, Bill Wickner continued his medical student research rotation in Gene’s laboratory. Bill had made significant progress in the purification of *E. coli* PS decarboxylase. Here was an integral membrane protein with a convenient assay that was readily solubilized in an active form by Triton X-100. Bill was a master at large-scale preparation. Membranes were isolated from a pound of *E. coli* cell paste followed by extraction and an acetone precipitation step. The first column was a 1-liter volume DEAE fractionation with a 10-liter salt gradient in Triton X-100 and 10% glycerol, which was collected in 250-ml fractions in a modified fraction collection. After a day in his clinical rotation, Bill would set this up in the evening on the lab bench behind mine. About 20% of the time, the fraction collector would malfunction, resulting in the floor becoming covered in the effluent, which I would have to clean up in the morning. After concentration of the decarboxylase peak and a gel filtration column, Bill had a yellow solution with a 500-fold increase in specific activity. I took over the project at this point because Bill was soon to join Arthur Kornberg at Stanford. As Bill was preparing his oral short presentation on partial isolation of a pyridoxal-dependent PS decarboxylase at the San Francisco meeting of the Federation of American Societies for Experimental Biology, I decided to subject the sample to a sucrose gradient centrifugation step. The result was a yellow pellet at the bottom of the tube and all the activity about halfway down the tube. We either had a newly discovered pyruvate-dependent decarboxylase or a long way to go before purity was attained. It turned out both were true. I completed the purification over several months starting with 8 kg of *E. coli* cell paste, which after a 3500-fold enrichment yielded about 14 mg of active nearly pure enzyme (10). I did not determine the identity of the prosthetic group; that would wait until Satre and Kennedy’s later work, identifying the group as pyruvate bound to a small subunit of the decarboxylase (24), although I did later return to this enzyme to study the mechanism by which the prosthetic group is generated (9). My tenure in Gene’s lab afforded the opportunity to study under one of the great scientists of the late 20th century, and form lasting personal and scientific friendships for the remainder of my career. Bill and I, with Gene’s careful guidance, had demonstrated that integral membrane proteins of phospholipid metabolism could be purified to homogeneity in an active form and characterized. This was a seminal accomplishment for the times and provided the foundation for the next 15 years of my research while stimulating others to carry out similar purifications in bacteria and eukaryotic cells.

Time to find gainful employment

1972 was not a great year to find a job. We were in the middle of the Nixon years of significant cuts in National Science Foundation (NSF) and National Institutes of Health (NIH) research support. Most of the new hires were concentrated in the Houston, Texas area, which was undergoing a renaissance in biochemistry. George Schroepfer moved from the University of Illinois to initiate the Department of Biochemistry at Rice University. Salih Wakil moved from Duke to Baylor College of Medicine and was increasing the Department of Biochemistry. Allan Goldstein came from New York to rebuild the Division of Biochemistry at the University of Texas Medical Branch in Galveston. John (Jack) DeMoss moved from the University of Californian at San Diego to chair the Department of Biochemistry and Molecular Biology at the newly formed University of Texas Medical School in Houston. I interviewed at all four institutions and decided to take my chances with Jack in building a new medical school.

When I interviewed in January of 1972, just after the birth of our son Michael during a December snowstorm, the medical school’s first building was a hole in the ground, with laboratories in rented space spread over the Texas Medical Center. When I arrived in August, the first two-story building, where
we taught the first entering class of medical students in September, was complete. The five founding members of the department were housed in cramped rented space on the 13th floor of the Center Pavilion Hospital where I shared an office and laboratory with Henry Strobel, who came from Minor Coon’s group. The building had previously been a hotel, and each room was converted to a shared office and laboratory. We were sandwiched between the hospital below and the drug halfway house on the floor above. The latter occupants would wander into our space and on one occasion collected bottles of radioactive toluene scintillation fluid in order to get high. That fall was spent writing our first grants, setting up our labs, organizing the medical school lectures, and designing small group conferences the week before each meeting with medical students. My first grant was funded on the initial pass in 1973 despite Nixon sequestering the NIH budget for a short time. One could still get a grant with a novel idea and a background to support the project without a lot of preliminary data. My paper on the PS decarboxylase was not published until 1974 (10). I had demonstrated the ability to purify at least one membrane protein in an active form and wished to purify and characterize a few more. Because the grant ran for 43 years, including a 10-year MERIT Award at the end, its original title of “Structure and Function of Membrane Proteins” proved to have unexpected longevity. Fortunately, after a short grant hiatus, the project currently continues under a new grant number. My recommendation has always been to pick a topic in an evolving area of research. Membrane proteins certainly fitted the bill at that time and remain an active area of research.

Jack was the model departmental chairman, which I tried to emulate during my chairmanship of the department in the early 1990s. Jack’s approach was to hire bright young faculty, provide strong financial support and guidance, and leave them alone to develop their unique niche in science. This tradition continues under the strong leadership of Rodney Kellems, the current chair of the department. I like to think that my decision to come to what is now the McGovern Medical School some 45 years ago and contribute to the research development, administrative leadership, and teaching mission of the school has in some small way contributed to the successful development of a strong clinical- and research-based institution.

When I was starting up, Gene had suggested I replicate my success in purification of a bacterial membrane protein in mammalian systems, and so I did spend a few months working with beef hearts and livers. However, my passion remained with E. coli lipid biosynthetic enzymes, and so I returned to this topic. This was a fortuitous decision as the explosion in modern molecular biology and genetics was about to occur. With my first graduate student Tim Larson and my first postdoc Takashi Hirabayashi, we set out to purify the E. coli PS and phosphatidylglycerol-P (PGP) synthases, the committed steps to major lipids of E. coli, phosphatidylethanolamine (PE) and PG/cardiolipin (CL), respectively (Fig. 3). Not wanting to repeat the difficulties of time and material I experienced with the PS decarboxylase, we capitalized on new approaches to protein purification by developing substrate affinity chromatography methods. For the PGP synthase, we covalently attached the lipid substrate CDP-diacylglycerol to a Sepharose column and eluted the enzyme with its substrate CDP-diacylglycerol (25). PS synthase tightly binds to phosphocellulose, which we specifically eluted using CDP-diacylglycerol (26).

Figure 3. Synthesis of native and foreign phospholipids in E. coli. Pathways native to E. coli are noted with solid arrows, and pathways resulting from foreign genes introduced into E. coli are noted with dashed arrows. The genes encoding the following enzymes and associated with each biosynthetic step are listed next to the arrows: 1) CDP-diacylglycerol synthase; 2) PS synthase; 3) PS decarboxylase; 4) PGP synthase; 5) PGP phosphatases; 6) CL synthases; 7) PG:MDO sn-glycerol-1-P transferase; 8) diacylglycerol kinase; 9) glucosyl diacylglycerol synthase (Acholeplasma laidlawii); 10) diglucosyl diacylglycerol synthase (A. laidlawii); 11) PC synthase (Legionella pneumophila); 12) PI synthase (S. cerevisiae); 13) O-lysyl PG synthase (Staphylococcus aureus). Figure originally published in Ref. 62 (Dowhan, W., et al. (2017) Functional roles of individual membrane phospholipid species in the mammal). With kind permission from Springer International Publishing.
Chris Raetz and I had a long and productive personal and professional relationship until his untimely passing from cancer in 2011, the same year Gene passed away. Upon completion of his M.D./Ph.D. at Harvard Medical School and a residency at the Peter Bent Brigham Hospital in Boston, Chris spent two years as a postdoctoral fellow with Herb Tabor at the NIH. While we toiled in the cold room purifying enzymes, Chris developed clever methods for isolating temperature-sensitive mutants in PS (27) and PGP (28) synthases followed by mapping of their encoding genes pssA and pgsA gene, respectively. The convergence of mutants in phospholipid biosynthesis and purification of functional biosynthetic enzymes occurred fortuitously with the emergence of molecular genetic engineering of E. coli. While at Stanford, Bill Wickner acquired the Clarke and Carbon collection (29) of E. coli DNA fragments carried on a ColE1 plasmid, which Bill sent to Chris at the NIH. Through complementation of the pssA mutant, Chris was able to clone the gene encoding PS synthase. We combined forces and used the overproduction of the PS synthase coupled with affinity chromatography to isolate about 20 mg of pure enzyme from 350 g of wet weight of E. coli cells (30). This represented one of the first uses of genetic engineering to obtain significant amounts of enzymes found in very low amounts. We later engineered 800-fold overproduction of the enzyme, which yielded 34 mg of enzyme from 20 g of cells (31).

In the mid-1970s to early 1980s, access to DNA ligase and a few restriction enzymes was very limited. I benefited by the arrival to our faculty of Terry Landers from Paul Berg’s lab and Charles McHenry from Arthur Kornberg’s laboratory. Terry brought the expertise and methodology for isolating several restriction enzymes, and Charlie purchased a 350-liter fermenter that we used to grow cells for isolation of enzymes for manipulation of DNA. With all these resources at hand, we cloned the pgsA gene and amplified its production for efficient purification (32). Using the tedious Maxam and Gilbert method, we sequenced the pssA (33) and pgsA (34) genes. Chris’s lab identified two genes (pgpA and pgpB) that encoded enzymes that dephosphorylated several phospholipids including PGP (35). However, double mutants still carried out PG and CL synthesis. We established that a third gene, pgpC, was responsible for anionic phospholipid biosynthesis (36). Chris later cloned pgpC and characterized mutants and the gene product (37). The combined efforts of mapping genes responsible for lipid biosynthesis, cloning the genes, and purifying the resulting gene products validated the lipid biosynthetic pathways delineated by our mentor, Gene Kennedy, and brought lipid metabolism into the modern age of molecular genetic engineering (summarized in Ref. 16).

In 1976, George Carman (Fig. 2) inquired about a postdoctoral position in my laboratory. His background was in food science, so he had a lot to learn about biochemistry, but what he lacked in expertise he made up for in enthusiasm and perseverance. We set out to apply surface dilution kinetic analysis to PS synthase to understand how a peripheral membrane enzyme carries out catalysis (38). Later George and Ed Dennis would provide a more detailed description of the method (17). George rapidly learned membrane biochemistry and enzymology. I am pleased that I was able to give George the opportunity to begin a very productive research career, which has resulted in him becoming one of the leaders in the study of lipid metabolism in yeast (39). My most significant contribution to yeast lipid studies was to convince George to stay in academics and turn down the opportunity to make instant gratin potatoes in the food processing industry.

A faculty leave develops new directions

In 1983, with the luxury of being a tenured full professor with two recently renewed NIH grants, I decided to take a faculty development leave for one year in Gottfried (Jeff) Schatz’s (Fig. 1) lab at the Biozentrum in Basel, Switzerland to be immersed in yeast genetics and mitochondrial bioenergetics. This would be a dramatic change in the direction of my research program that would also benefit my entire family. To get a head start, I attended the Cold Spring Harbor yeast genetics course in August before moving to Basel. Armed with funds from a Guggenheim Foundation fellowship, my NIH grants, and my institutional support, we arrived in Switzerland in September, 1983. Our son Michael attended 6th grade in the International School of Basel. He developed his French and a great sense of independence through taking the bus and streetcar every day to school. Our son David attended the 10th grade at Aiglon College, an English curriculum boarding school in the Swiss Alps city of Chêseres-Villars on the east side of Lake Geneva; winter physical education included daily skiing. My wife Jerilyn, the kids, and I enjoyed trips throughout Europe and 2 weeks in Egypt. Although there are many personal and professional obstacles to sabbatical leaves, I can strongly recommend such an experience if you have the opportunity, as it enriched our personal lives and my professional career. I also took development leaves in 1991 and 2010 in Robert Simoni’s laboratory at Stanford University.

Jeff Schatz was a widely read Renaissance scholar and great intellect. Besides his seminal studies in mitochondrial bioenergetics, Jeff was an accomplished violinist and a member of the Basel orchestra. During my stay in the lab, I honed my skills in yeast genetics, was introduced to the ease of making gene knockouts in yeast, and acquired a strong background in mitochondrial bioenergetics, which would change the direction of my research once I returned to Houston. The knock-out I constructed in the nuclear COX4 gene that encodes a subunit of the mitochondrial cytochrome c oxidase respiratory Complex IV resulted in failure of the multiple subunits of the complex to assemble (40). I tried to get Jeff interested in yeast lipids, in particular the role of CL in mitochondrial function. However, Jeff, like many at the time, felt that lipids were not important because the purified respiratory complexes were fully active in detergents. My own interest in this question remained, and so I returned to this system in later studies on the role of CL in mitochondrial function, discovering for example that mutants lacking PG and CL in the mitochondria fail to translate the COX4 gene mRNA, thus demonstrating communication between the mitochondria and the nucleus (41).

While I was in Gene Kennedy’s lab, one of our weekly research meetings discussed Karel Wirtz’s report of a mamma-
lian cytoplasmic protein (phosphatidylcholine (PC) transfer protein or PCTP) that catalyzed the exchange of PC between membranes (42). Later a similar activity (PITP) that exchanged phosphatidylinositol (PI) and PC between membranes was reported (43). These were hypothesized as being involved in moving phospholipid from the site of synthesis in the endoplasmic reticulum to other cellular membranes. While in Basel, I initiated a partial purification of the latter PITP activity from *Saccharomyces cerevisiae*, which formed the basis for an NIH proposal. Shortly after receiving funding upon my return to Houston, Paul van Heusden joined my group from Wirtz’s lab and, along with a graduate student, Jackie Aitken, continued the purification. To my dismay, their first attempt separated the transfer activity from the protein I had incorrectly proposed as the PITP. However, persistence resulted in purification of the fraction containing PITP activity to homogeneity, and by using the partially determined protein sequence, the encoding gene was identified (44). This paper was recognized as a Journal of Biological Chemistry Classic (45). Shortly after publication of the results, I was contacted by Vyta Bankaitis, who had been working on the Sec14 protein, which Randy Schekman had identified as a component necessary for movement of proteins from the endoplasmic reticulum to the Golgi of yeast. We collaborated to demonstrate that Sec14 and PITP are one and the same (46). This collaboration opened up the whole area of studies pioneered by Bankaitis’ lab on related phospholipid transfer proteins, which turned out to be regulatory proteins that integrate various cell processes with lipid metabolism.

**Why are there so many lipids?**

When I returned to Texas, I also returned to studies of phospholipid genetics and function in yeast and *E. coli*. However, my exposure to yeast genetics changed the course of my research from enzymology and protein chemistry to addressing the role of lipids in cell function. I was fortunate to hire Phil Heacock, a very bright and resourceful technician who would be the primary support of our molecular genetic manipulation of yeast and *E. coli* over the next 20 years. Outside of the emerging area of lipids and their derivatives as second messengers, lipids were still viewed as merely the hydrophobic medium in which membrane proteins are embedded. But if a single phospholipid can form a lipid bilayer, why should there be so many lipids (47)?

We set out to make null mutants at the branch point of phospholipid biosynthesis, i.e. in the pssA and pgsA genes (Fig. 3). This was before the advent of the elegant and efficient methods available today to carry out genetic manipulation and counterintuitive because Chris Raetz had shown that conditional mutations in these genes were lethal. However, I noticed that the temperature-sensitive mutants *pssA* and *psd* were viable, with low PE or high PS and low PE, respectively, if grown at the restrictive temperature in the presence of millimolar concentrations of Mg\(^{2+}\). Extensive analysis of the phenotypes of the Δ*pssA* mutant, which is viable if grown in the presence of millimolar levels of Ca\(^{2+}\), Sr\(^{2+}\), or Mg\(^{2+}\) (34), has provided fertile research projects to the present day. Although PE is not absolutely required under controlled growth conditions, lack of PE results in rapid cell lysis in the absence of divalent cations. Under viable conditions, the mutant displays defects in cell division, energy metabolism, and structural organization of most secondary solute transporters (16).

Under the conditions we used, the ΔpgsA strain we initially constructed was not viable, suggesting that PG is essential (48). The ΔclsA strain, lacking what turned out to encode the major CL synthase, was still robustly viable. Later Raetz’s group, with a little help from us, characterized deletions in the remaining two clsBC genes, which encode additional CL synthases, and demonstrated that cells completely lacking CL are viable (49). One could bypass the need for PG specifically by eliminating the major outer membrane lipoprotein (encoded by *lpp*) that derives a glycerol moiety from PG. The glycerol forms the diacylglycerol in the thioether linkage to the N-terminal cysteine that tethers the protein to the inner leaflet of the outer membrane. Failure to make this link results in accumulation of the nascent protein in the inner membrane, resulting in cell death. However, the ΔpgsA Δlpp strain remains temperature-sensitive for growth (50). Growth at high temperature was a step we used during the final steps of constructing the ΔpgsA strain, leading to the conclusion that cells could not survive without PG. These additional properties of the mutant were eventually sorted out, resulting in a viable strain that was used to probe additional roles for anionic lipids in cell function.

Ascertaining functional roles for lipids at the molecular level poses several obstacles. Many early conclusions of lipid function were based on the effects of lipids added to *in vitro* studies of cell processes. However, little attention was paid to the complex interplay of physical and chemical properties of lipids — defined by both the chemistry of the hydrophilic head groups and the physical properties of their diverse fatty acid composition — thus leading to artifacts. Because genes encode the lipid metabolic enzymes and not the lipids directly, mutations affecting biosynthetic pathways are indirect in defining function. Loss of function can be highly pleiotropic, especially in eukaryotic cells with multiple membranes. Elimination of a major lipid may result in loss of cell integrity before a direct requirement in a cell function is revealed. Our approach has been to generate viable but compromised mutants with altered lipid composition followed by detailed characterization of phenotypes in cells. In order to establish a direct lipid-cell function relationship, the phenotypes have been reconstituted in well-controlled *in vitro* studies. This approach has resulted in defining at the molecular level previously unrecognized roles for lipids in such diverse processes as membrane protein folding, DNA replication, cell division, protein translocation across membranes, energy transduction, and organization of phospholipids into domains (16).

In 1991, shortly after construction of the above null mutants, two very talented postdoctoral fellows joined my group. Mikhail (Misha) Bogdanov focused on the ΔpssA strain properties, and Eugenia Mileykovskaya initially characterized the ΔpgsA strain. Both have become important faculty collaborators. Eugenia developed earlier findings showing that, as in eukaryotic cells, membrane phospholipids are not evenly distributed laterally in the inner membrane bilayer of *E. coli*. Using 10-­N-nonyl acridine orange (NAO), which binds spe-
A growing role for yeast

Following our success in E. coli, we extended the approach to S. cerevisiae. Influenced by my experience in Basel with mitochondrial function, we focused on genes responsible for the synthesis of PE, PG, and CL in the mitochondria. I chose yeast for these studies because cells compromised in mitochondrial function can still be grown on glucose. Aided by an M.D./Ph.D. student Shao-Chun Chang and a postdoctoral fellow Constance Clancy, the yeast nuclear genes PSD1, PGS1, and CRD1 that encode the mitochondrial Ps decarboxylase (53), PGP synthase (54), and CL synthase (55), respectively, were cloned and knocked out. The PSD1 gene was cloned by complementation of the E. coli temperature-sensitive mutant in the Ps decarboxylase. The PEL1 gene had been cloned and was reported to be a second PSS (CHO1) gene. We noted that the null mutant lacked PG and CL, and thus surmised correctly that it was the PGS1 gene. The null mutant turned out to be respiratory-deficient and displayed structurally aberrant mitochondria. The CRD1 gene was uncovered during our initial attempts to find the PGS1 gene by homology searches using the E. coli PGP synthase sequence, which like the yeast CL synthase, contains motifs that recognized CDP alcohols such as CDP-diacylglycerol. The Δcrd1 strain accumulates PG up to 20% in the mitochondria in place of the normal 20% CL. Surprisingly, PG appears to partially substitute for the lack of CL in respiratory function, which was unexpected given the exclusive localization of CL to the mitochondria.

After extensive studies on the function of anionic phospholipids in E. coli and at the beginning of the new millennium, Eugenia and a talented graduate student, Mei Zhang, focused on the role of CL in mitochondrial function (summarized in Ref. 56). Although Chance postulated in 1959 (57) that the individual mitochondrial electron transport chain complexes were organized into a higher-order supercomplex or respirasome, our initial membrane insertion as well as in governing dynamic structural organization of membrane proteins post-assembly. In wild-type cells, as predicted by the Positive Inside Rule, membrane proteins assemble with positively charged extra-membrane domains facing the cytoplasm. Those domains that are negatively charged or neutral face the trans side of the membrane. The Charge Balance Rule is an extension of the Positive Inside Rule that incorporates the effect of membrane lipid composition in determination of the orientation of transmembrane domains with respect to the plane of the lipid bilayer. The in vivo experiments carried out by Misha and me demonstrate that the membrane lipid composition has a direct effect on topological organization of membrane proteins at the time of initial insertion of transmembrane domains and protein folding (Fig. 4). The presence of PE suppresses the membrane translocation potential of negative residues in extramembrane domains containing a mixture of oppositely charged residues. However, in the absence of PE, negative residues become strong topological determinants, resulting in a change in orientation of neighboring transmembrane domains. Most dramatic is that topological organization is not fixed at the time of initial folding.
but can be changed post-folding by a change in lipid environment. The effect of PE and other net neutral or positively charged lipids is to dilute the high membrane negative surface charge due to PG and CL. Therefore, membrane protein structural organization remains dynamic and responsive to changes in the local lipid environment. Heidi Vitrac, who joined our research group recently, reconstituted the dynamic properties of membrane proteins in a totally in vitro system composed of only LacY and lipids. She established that the rate of topological re-arrangement occurs on a time scale of seconds, making such changes of biological significance for any protein in any membrane potentially independent of other cellular factors (60). She also demonstrated that phosphorylation of a membrane protein extramembrane domain, which alters the charge balance between the protein and the membrane surface, results in a similar rapid change in topological organization (61).

The above studies were carried out using primarily LacY as a model 12-transmembrane domain-spanning protein, which again illustrates how early studies have influenced my current focus. None of the progress made on the role of phospholipids in determining membrane protein topological organization would have been possible without the pioneering work of Ron on LacY and his willingness to provide us with LacY derivatives and advice. Our current studies are focused on defining in molecular terms how membrane lipid composition and protein phosphorylation govern the dynamic structural organization of membrane proteins.

In 2005, Christian Raetz nominated me as the recipient of the ASBMB Avanti Award in Lipids. Walter Shaw, president of Avanti Polar Lipids, presented the ASBMB Avanti Award in Lipids to me in San Diego, California in April of that year (Fig. 5).

In 2014, my former postdoctoral fellows George Carman and Weiming Xia organized a William Dowhan Symposium. This
was attended by former lab members, collaborators, and members of the Texas Medical Center community in Houston, Texas in April of that year (Fig. 6).

Concluding remarks

My scientific career has been continually focused on understanding how proteins interact with their lipid environment to attain final and dynamic structural organization. There have been many recurring themes throughout my career. Early exposure to the principles of hydrophobicity, protein chemistry, LacY, membrane proteins, lipid metabolism, molecular genetic manipulation, and yeast mitochondria have greatly shaped and directed my career. I chose not to follow the crowd but to challenge dogma, convinced that lipids do more than form bilayers and that detergents do not fully replace lipids. Fortunately, with a good amount of luck, I have been right more than I have been wrong. Finding conditions where null mutants in lipid biosynthetic enzymes compromised cell function without destroying membrane barrier properties was fortunate. Although most secondary transporters require PE for full function, only a subset, including LacY, is dependent on PE for proper native topological orientation. The fortuitous selection of LacY as our initial model membrane protein lead to the discovery that fully folded proteins can undergo topological inversion. The guiding principle has always been to understand mechanisms at the molecular level by following up genetic alteration of cell function with detailed in vitro studies. As with all successful endeavors, progress has relied on contributions from many collaborators. I was fortunate in my early years to pursue science at a time when curiosity research was highly valued and financial support could be gained based on novel ideas that addressed understanding basic mechanisms in living systems. I hope today's scientists can get the same chance to follow their curiosity.

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