Stephen H. White

ANNUAL REVIEWS

Annual Review of Biophysics

Fifty Years of Biophysics at the Membrane Frontier

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Annu. Rev. Biophys. 2023. 52:21–67

First published as a Review in Advance on February 15, 2023

The *Annual Review of Biophysics* is online at biophys.annualreviews.org

https://doi.org/10.1146/annurev-biophys-051622-112341

Keywords

membrane structure, membrane proteins, membrane protein biogenesis, lipid bilayers, lipid–protein interactions, x-ray and neutron diffraction

Abstract

The author first describes his childhood in the South and the ways in which it fostered the values he has espoused throughout his life, his development of a keen fascination with science, and the influences that supported his progress toward higher education. His experiences in ROTC as a student, followed by two years in the US Army during the Vietnam War, honed his leadership skills. The bulk of the autobiography is a chronological journey through his scientific career, beginning with arrival at the University of California, Irvine in 1972, with an emphasis on the postdoctoral students and colleagues who have contributed substantially to each phase of his lab's progress. White's fundamental findings played a key role in the development of membrane biophysics, helping establish it as fertile ground for research. A story gradually unfolds that reveals the deeply collaborative and painstakingly executed work necessary for a successful career in science.

Contents

PROLOGUE

It was a dark and stormy night in Pavia when Jackie and I arrived at *Al Cassinino*. We were greeted by the owner, Agostino Cremonesi, who runs the restaurant in the traditional Italian manner. His wife runs the kitchen, and their children, Chiara and Stefano, manage the elegant dining room filled with stunning antique furniture and dinnerware. As we departed after a wonderful evening of amazing dining, we told Agostino how much we enjoyed the food and ambiance. He paused for

a moment, struggling for the right words in English. Then, with hand over heart, he said, "I must do it!"

I understood exactly what he meant! I have felt the same way about science since my childhood years in the Jim Crow South. Whatever my lab has achieved toward understanding the fundamental principles of membrane structure and assembly, I owe much to my supportive father, to generous mentors, and especially to collaborations with postdocs and colleagues with whom I have shared the adventure of discovery in membrane biophysics. This autobiography is the backstory of that adventure.

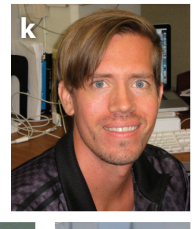
A YOUNG KID IN THE SOUTH

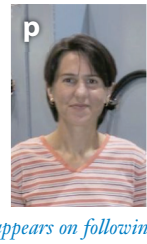
My father, James Halley White (**Figure 1*a***), was a public health physician who established the first health department in Lowndes County, Mississippi, in the 1940s. His early years were spent in Long Beach, California, but his coming-of-age years were spent in Oklahoma. He worked his way through college by working in the oil fields as a horse-mounted pipeline rider. He eventually earned an M.D. degree from the University of Oklahoma and practiced medicine in the tiny oil boom town of Wewoka. This was during the Great Depression, which meant delivery of health care by Model T and payment in chickens and potatoes.

In the late 1930s, he was recruited by the US Public Health Service to train as a health officer to establish the Lowndes County Health Department in Columbus. This was part of the effort of the director of Mississippi Department of Public Health, Dr. Felix Underwood, to establish health departments in all 82 Mississippi counties. In 1941, only 56 counties had county health departments (14). The conditions under which Southern poor lived are unimaginable today, and the health care available for poor Black or White tenant farmers—but especially if you were Black—was atrocious (125). Health care was provided mainly by ill-trained Black midwives until 1926, when the first Black registered nurse was appointed by the board of health (104, 112, 123). Black nurses became the key to improving Black health care, beginning with the training of midwives (2).

Dad's training in public health began in the Mississippi Delta in Indianola, Sunflower County, where a training facility was established following the great Mississippi River flood of 1927. Sunflower County was also home to the notorious Parchman Plantation work farm, which gained infamy in the 1960s as the place of incarceration of the Freedom Riders. My dad, mother, and sister Sally moved to Columbus in late 1939 or early 1940, when he was establishing the Health Department. Because their lives were unsettled during this period, my mother returned temporarily to Oklahoma to be with family while birthing me. I was born in Wewoka in May of 1940. [Wewo-ka is a Seminole word translated as "barking waters" that described a small waterfall north of the town (140).] It was one of the end points of the Trail of Tears traveled by the Seminole Indians when they were forcibly removed by the US government from Georgia, Tennessee, Alabama, North Carolina, and Florida in 1838.

I often reflect on growing up in the Jim Crow South, especially today in the light of the Black Lives Matter movement. Memories of those days are vivid ones: Whites-only drinking fountains, segregated schools, and all the rest. Most middle-class prepubescent white kids in the South were not formally taught about the "color line." We, at least my sister Sally and I, were just naïve observers of the human condition, too young and ignorant to understand what was going on. The only way I could glimpse the consequences of segregation was from the attitudes and behavior of my parents. They implicitly sensitized me in three ways. The first was that they never used the "N word." The second was their expectations about my interactions with my "mammy," Irene Wilkens. She didn't just work for us; she was a member of the family. Irene came to work every morning to make breakfast, keep house, care for me when I got home from school, and get dinner





(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Family album. (*a*) Me and my dad, James Halley White, 1943. (*b*) My children and I, 1996. Clockwise: Sharr, me, Shell, Sunde, Skye, Saill, Storn. (*c*) Asilomar meeting with Ken Dill Lab, 1983. Left to right: Jay Edelman, Ken Dill, Bob Cantor, Russ Jacobs, Glen King, and me. (*d*) My 65th Birthday Symposium, 2005. Left to right: Alex Ladokhin, me, Kalina Hristova, Bill Wimley, and Mike Wiener. (*e*) TEMPO group, 2004. Front row, left to right: Mike Myers, me, Doug Tobias, Craig Snider. Second row: Hirotaka Sasaki, Mónica Fernández-Vidal, Simon Jaud, Alfredo Freites. Back row, left to right: Francisco Castro-Román, Ryan Benz, Alex Ladokhin, Ella Mihailescu. (*f*) With Nicoletta Bondar at my 65th Birthday Symposium, which she organized. (*g*) Blanco Lab at the Physiology Department Retreat, 2019. Left to right: Craig Snider, me, Eric Lindner, and Guillaume Roussel. (*h*) Gunnar and I at Stockholm University celebrating my honorary Ph.D., 2008. Note the laurels headdress! (*i*) Jack Rush and I celebrating the commissioning of the Advanced Neutron Diffractometer/Reflectometer at NIST Center for Neutron Research, 2006. (*j*) Gargi Dasgupta, my lab manager. (*k*) Magnus Andersson. (*l*) Martin Ulmschneider. (*m*) Mónica Fernández-Vidal. (*n*) Sara Capponi. (*o*) Sajith Jayasinghe. (*p*) Ella Mihailescu.

started. (Whatever my mother knew about cooking, she learned from Irene.) I was about five years old, playing at blocks, when Mother came into my room to tell me to make my bed. I snottily said, "Let Irene do it, that's what we pay her for." Bad consequences for me, but an important lesson in respecting and valuing people regardless of race or social status. (And I still make my bed every morning!)

The third, which was key, occurred when my father and his chief nurse Cary, who was Black, were planning one of many immunization campaigns. He visited her at her home one evening to deal with some administrative issue. When he returned to the car, where I was waiting, he said, "Cary has a beautiful home. It's too bad she can't paint it to look nice from the outside." What!? Why not? He said, "If her house looked nice from the outside, people would think she was 'uppity'." That's when the meaning of Jim Crow began to sink in.

MY CRAFTSMAN DAD

Dad once told me that rather than being a physician, he wanted to be an engineer. But during the Depression, jobs for engineers were scarce, leading him to medicine. He loved making things—toys for me and furniture for the family. He made a solid cherry desk for Sally using lumber that he picked up from a farmer who had used two-inch-thick cherry planks to patch the deck of a short bridge. For me, he made a bunk bed from gum tree planks taken from the steeple of a 100-year-old church that was being demolished. I spent hours watching him work and helping feed boards through the table saw. His workshop was complete with all the requisite power tools, which he showed me how to use safely. As I grew older (8–10 years), he told me I could use the shop anytime I wanted, but I had to clean it up when I was finished. This permission was important to me; he trusted me to be careful with dangerous machines to build stuff. Later he bought for me a No. 10 Gilbert Chemistry Set that I used without supervision. Can you imagine a parent doing that these days? A methanol burner for heating test tubes filled with dangerous chemicals? Horrors! A 10-year-old kid operating a band saw unsupervised? Call in the child welfare agents!

A KID OUT OF THE SOUTH

Around 1950, Dad was recruited to become Carter County Health Officer in Ardmore, Oklahoma. We were there only for a year. The problem was that the county health officer reported directly to the county commissioners, one of whom owned a truck dealership, and the law required that the purchase of garbage trucks be approved by the county health officer. Because Dad did not recommend the brand of truck preferred by the county commissioner, a huge political explosion ensued. Dad was a person of principle and refused to back down. I remember going into stores and

having people tell me to tell my dad that they were pulling for him. After a year of suffering under Oklahoma old-boy politics, we moved to Greeley, Colorado, where Dad had accepted the position of Weld County Health Officer. The health officer reported to a politically independent board that insulated him from county commissioner politics. You can be sure that Weld County had health officer–approved garbage trucks! The transition from the Jim Crow South to Colorado was a dramatic cultural shift that severed us cleanly from Southern culture just when *Brown v. Board of Education* was being decided. The biggest personal change for me at the time was that I forced myself to give up my Southern accent to avoid harassment from my schoolmates!

THE LURE OF SCIENCE

I was about 13 when Dad decided I should have a part-time job to keep me from getting up to no good with junior high school pals. So I went to work at Campus Pharmacy as a stock clerk and, in summers, as a soda fountain clerk. I guess I was a good worker, because the owner of the pharmacy offered to send me to pharmacy school when I graduated from high school. Count pills for a living? No thanks! The small hourly wage from that job allowed me to support my interest in astronomy and photography. I built a dark room in the basement for developing film from a camera I built for the 4.5" reflector telescope constructed from a cardboard linoleum tube. Later I built a better one using an aluminum irrigation pipe. Its hot feature was a rack-and-pinion focusing device I built from some war-surplus gears.

When I turned 16, the legal driving age in Colorado, Dad agreed that I should have a car. I found one in the classified ads: a 1947 black Ford Coupe listed at $125. That was a lot of money for a kid to come up with. Dad could have easily given or loaned me the money. Instead, we went down to the bank, and I borrowed the money with Dad as the guarantor. He was teaching me to be responsible by expecting me to be so.

Dad bought our first television set when we were living in Mississippi. Because we were 100 miles from the nearest broadcasting station in Birmingham, the picture was generally quite "snowy" due to the weak signal. So, Dad set about building ever-more-sophisticated Yagi antenna arrays, the ultimate one being a channel 13 array consisting of 16 Yagis built from copper tubing. There were no TV repair shops then, but Dad found a Navy veteran who had been a radar operator during the War who knew how to adjust the electronics for optimal performance. One of the happy events for us when we moved to Colorado was that we were only 50 miles from the Denver TV transmission sites on Lookout Mountain. Great reception!

With this background, it was natural that we would become interested in ham radio. Together, we earned first Novice and then General Class amateur licenses, which required understanding electrical circuits and proficiency in Morse code. For those interested in such things, I am ex-KØEPF and ex-W6RKI. We built transmitters from scratch and shared the fellowship of pals in the local amateur radio club. By high school, I had become proficient enough in electronics to earn a commercial Second-Class Radiotelephone License, which is required by the FCC for servicing commercial transmitting equipment. In those days, long before the internet and cell phones, ranchers and truckers communicated over the vast prairie lands by "two-way radio." I worked for two outfits that sold, installed, and maintained two-way radio systems, which required the construction of 150-ft radio towers. So there I was, a 17-year-old kid driving all over northeastern Colorado and southwestern Nebraska, leading a crew of three guys installing and servicing commercial radio systems, including the towers.

A BUDDING SCIENTIST

At the ham radio club, I met Robert Lyon, who was studying mathematics at Colorado State College of Education (now the University of Northern Colorado). He was also a ham radio operator

who had been a Navy radio specialist and was supplementing his GI Bill income by servicing commercial radio systems. Before I started doing the same, I drove all over eastern Colorado with him to keep him company. He was the first real intellectual I knew. He was interested in everything. We talked about all sorts of stuff and had lots of fun, and hamburgers at various mom-and-pop greasy spoon restaurants along the way. He had a profound influence on me as I was finishing high school and starting to think about college. He felt, of course, that I should be a mathematician.

One very cold, crispy clear evening in Colorado as I was driving home from hanging out with pals, the radio program I was listening to was interrupted by a news flash: The Russians had launched Sputnik! Suddenly, high school kids interested in science were directed by school counselors toward engineering in college. Dad was excited about this and thought I should study engineering, even though I was more interested in physics. Our compromise was engineering physics, in which I enrolled as a freshman in 1958 at the University of Colorado (CU) School of Engineering in Boulder. The main difference between "pure" physics and the engineering variety was the elective courses (e.g., strength of materials for engineers, political science for the Arts & Sciences types). My first year was a disaster; I came close to flunking out and realized, with the help of guidance counselors, that I was not suited for engineering. Although my various tests suggested I was most like a chemist, I switched majors to "pure" physics. I guess the tests were pretty accurate, because much of my research work can be considered that of a physical chemist. The switch in majors caused me to lose a year to complete the Arts & Sciences requirements, one of which was biological science. Olwen Williams taught the course. She was a terrific lecturer. The lecture I remember most vividly was the one on the nerve action potential that summarized the Hodgkin-Huxley model. Wow! Physics has direct applications in biology! I tucked that away in my mind for future reference.

The introductory "sophomore physics course" for engineers and scientists was taught by Walter Tantilla. Besides being an excellent teacher, he was something of a character, with a droll sense of humor. He grew up on a family farm in northern Minnesota, the youngest of 12 children. An older brother had died in Spain fighting fascism during the Spanish Civil War. I approached him after one his lectures, told him I knew how to build electronic equipment, and asked if I could work in his nuclear magnetic resonance (NMR) lab. Sure, he said. And so, I became involved with his research and that of Seth Mizushima.

I spent a lot of time in the lab, building equipment and hanging out with graduate students and postdocs. My love of science blossomed. Eventually, Seth gave me my own project, which was to build a liquid-state laser based on naphthalene. I was appointed as an undergraduate research assistant, which helped pay for my last year of college. The laser project was a particularly thrilling one at the time because of the great excitement surrounding the invention of the ruby laser, reported in 1960 by Theodore Maiman (90), who had earned a degree in engineering physics at CU. Although the naphthalene laser was doomed to failure because the triplet state we were pumping into was too long-lived to lase effectively, the experience of being trusted with a project of my own was important. Even today, the Physics Department at CU is a haven for budding young physicists; it encourages and supports them to carry out original research.

BECOMING A SCIENTIST

The military draft was a real threat to young college men in the 1960s; being drafted after graduation was common. Because my father had served in the National Guard as the medical officer of the 31st Division artillery, two years of active service as a commissioned officer seemed preferable to being drafted, and I joined the Army ROTC. A particular benefit of continuing through four years of the program was being paid during the last two years of training. The Army unit at CU was an engineering unit, meaning that upon graduation I would be commissioned a second

lieutenant in the Corps of Engineers. For those continuing to years three and four, six weeks of basic training at Ft. Leonard Wood, Missouri, was a requirement. Besides the standard basic military training, we learned how to build bridges and roads—and even drive caterpillar tractors. Except for the sweltering heat of summertime Missouri, it was great fun.

Despite active duty in the Army looming, original research was what I wanted to be doing more than anything else. Vietnam was nothing more than a twinkle in the eyes of Kennedy and McNamara, which made it easy to obtain a Delay of Entry on Active Duty to attend graduate school before serving my two years. My application was accepted after I applied to the University of Washington graduate program in physics to pursue a Ph.D. Walt Tantilla, who had received his Ph.D. there, wrote a strong letter of recommendation that helped my acceptance despite my poor performance as an engineering student.

I arrived in Seattle in September of 1963. Because of my extensive hands-on research experience, I was given a graduate research assistantship in the lab of Hans Dehmelt, working on the radio frequency spectrum of the hydrogen molecule ion. Hans and his colleagues were extraordinarily clever experimentalists. I built equipment, electronic and mechanical, for the experiment under the direction of one his postdocs. I attended classes in the morning, worked in the lab every afternoon, and solved physics homework problems at home in the evening. It was an exhausting schedule. As time passed, I worried about my future in physics. I liked understanding new ideas and concepts, but I found the gory details of Green's functions and such uninteresting. Once I understood a principle, I saw no point in beating it to death. I knew I would never succeed as a theoretical physicist, and I did not want to be an instrument scientist working with a large team on particle accelerators. I liked the idea of working on a project that I could, literally, wrap my arms around.

After hearing a lecture by Arthur Brown from the Department of Physiology and Biophysics (P&B) on cell membranes, my interest in biophysics was reignited. The field was in its infancy, and I thought I might be able to do some interesting science and make fundamental contributions. So, I took some courses in P&B and transferred to their graduate program. I never would have done that if Hans had said to me, "Look, White, what I want to do is trap a single electron!" That was what he ultimately did, and he shared the 1989 Nobel Prize in Physics. I would certainly have earned a Ph.D. in physics had he shared that dream.

HEAD FIRST INTO MEMBRANES

In 1952, Hodgkin and Huxley had reported their voltage-clamp studies of squid axons, defining the sodium and potassium currents that flowed across the membrane during the propagation of the action potential. They received the Nobel Prize in Physiology or Medicine in 1963 for the work that Olwen Williams had lectured about so brilliantly in my biological sciences class. As a result, I was drawn to electrophysiology and measurements of transmembrane (TM) potentials. Walter Woodbury in P&B, who had made the first in vivo recordings of action potentials in live cardiac tissue, had a deep and intimate understanding of Hodgkin and Huxley's work. I chose him as my graduate mentor and began microelectrode measurements of resting membrane potentials of frog sartorius muscle fibers, in collaboration with my graduate student pal Michael Mackey. Walt taught a general course on membrane biophysics and a specialty course on the Hodgkin and Huxley papers, which we studied in exhaustive detail. Walt was also an expert in acid-base chemistry and was interested in the bicarbonate permeability of muscle cells. Mike and I worked together on the project, assembling an intracellular recording system. We spent many hours engaging with Walt on all aspects of electrophysiological measurements, including how to "pull" microelectrodes by hand.

Walt was a marvelous mentor who did not press us to work on problems of particular interest to him. Rather, he gave us the leeway to pursue whatever we found interesting. And there was a lot to be interested in! At the time, there was only speculation about the structure of cell membranes; the lipid bilayer as the organizing principle was controversial. How ions crossed membranes was unknown. One idea was that phospholipid headgroups gated ion flow through lipid bilayers. If there were channels, were there separate ones for potassium and sodium? The idea that the hypothetical ion channels were proteins was wild speculation. Recall that there were still only a few proteins, soluble ones, whose structures had been determined by the end of the 1960s. I decided that to understand how ions might cross membranes I needed to understand everything I could about cell membranes, their proteins, their lipids, and particularly their structure. I spent many intense hours, days, and weeks in the library reading everything I could about membrane structure as a first step toward understanding ion permeation.

There were some speculative and crazy ideas about membrane structure at the time, but the literature generally favored the idea of the lipid bilayer as an important structural element: Was it a lipid bilayer coated with proteins (24) or a sheet of protein subunits coated with lipid (36)? I finally decided it must somehow be a combination of the two, which I proposed in a review article written with Walt, Mike, and our colleagues (184) (**Figure 2**). That was my first more-or-less original idea about membrane structure, and it hinted at the fluid-mosaic model (121). During my reading, I came across papers by Paul Mueller and coworkers (93) and Denis Haydon & Janet Taylor (38) showing that a single lipid bilayer could be formed across a small hole drilled through an inert barrier such as polyethylene or teflon. As a result, there was little doubt in my mind that the bilayer was the organizing structural element. Just think about it: a lipid film two molecules thick! I was hooked. Mike Mackey and I were intrigued and immediately set about making a bilayer chamber, a Wheatstone bridge to measure electrical impedance, and a camera-equipped dissecting microscope to determine photographically the membrane area (141). That was the starting point for my 1969 Ph.D. dissertation, "The Specific Capacitance of Black Lipid Membranes," in which I showed that applied electric fields could compress and thus change the thickness of the films (142).

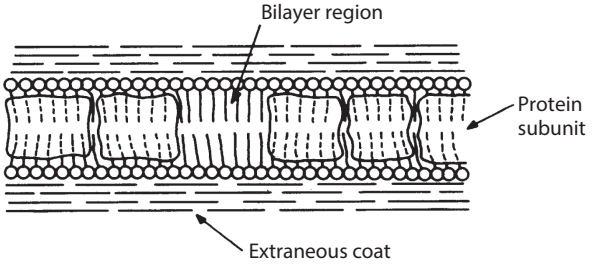


Figure 2

Composite model of cell membranes (1968). The model summarized ideas about membrane structure in 1968 (184). There were three opposing models. The most often presented one was a lipid bilayer with "extraneous" coats on the surface, which were suggested to be mucoproteins and mucopolysaccharides or denatured protein (24, 105). Another proposed a protein matrix incorporating lipid micelles (88). Finally, David Green's subunit model suggested that membranes were composed of protein subunits that aggregated into sheets due to selective binding of lipids to certain surfaces of the subunits (36). The idea was that lipids adsorbed to some surfaces to interrupt protein–protein interactions, causing the formation of protein sheets. My composite model assumed that Green's subunits could form islands within lipid bilayers, hinting at the Singer & Nicolson (121) model. Figure adapted from Reference 184.

TOM THOMPSON

In the meantime, the US Army was keeping track of my progress as the Vietnam War escalated. Driving to the lab one night in January 1968, I heard news of the beginning of the Tet Offensive, which told me that my Delay of Entry on Active Duty would soon end. Sure enough, in the summer of 1968 I received orders to report for active duty in March 1969. I had learned earlier that John Bateman, a senior biophysics investigator at Ft. Dietrick, Maryland, had an opening in his laboratory and would love to have a person with a Ph.D. in biophysics. The Army subsequently transferred me to the US Army Chemical Corps and assigned me to Ft. Dietrick as my permanent duty station. But first, I was required to undergo training as a chemical, biological, and radiological defense officer at Ft. McClellan, adjacent to the city of Anniston, Alabama. I was being returned to the South by the Army, not too far from Columbus, Mississippi! On the way to McClellan, we stopped in Columbus briefly to renew old friendships, and particularly to see Irene. Sadly, she had passed away the night before I arrived.

After six weeks of tramping around in the Alabama woods learning how to be an officer and gentleman, I headed to Ft. Dietrick, which is only about 100 miles from the University of Virginia (UVa), where Tom Thompson was Chair of the Biochemistry Department. I called and asked if I could visit on my way to Ft. Dietrick. The Biochemistry Department was a membrane biophysics hot spot at the time, and Tom was one the leading figures along with Ching-chin Huang and Rod Biltonen. After I presented an informal seminar on my black film work, Tom inquired about my assignment to Ft. Dietrick. He surmised that I would not have a choice about research projects and suggested it would be great if I could somehow spend part of my time in Charlottesville. At that moment in history, the Chemical Corps and Ft. Dietrick were taking heavy fire from political activists of all stripes; busloads of antiwar demonstrators camped out in Washington, DC, during the weekends; and Nixon was busy negotiating the SALT talks with the Soviets. It seemed clear that Dietrick would be under heavy pressure from the public and Congress to give up biodefense work. To help burnish their image, the managers at Dietrick were delighted to have me collaborate with folks at UVa. They even agreed to assign me there on temporary duty every few months and to pay my travel expenses.

Tom found a small lab space for me and generously equipped it with all the stuff I needed to continue my black film work. He didn't care what I worked on but thought whatever I wanted to do would be worth supporting. My dissertation work had been carried out using oxidized cholesterol membranes (142), which were extraordinarily stable but were a chemical mess. The recipe involved bubbling oxygen through a cholesterol–decane mixture overnight under gentle heat! My goal was to do high-precision measurements of specific capacitance on black films formed from very pure phospholipids with known acyl chain structures. Tom's group was expert in phospholipid chemistry and purification, which allowed me to learn all about lipid extraction, thin-layer chromatography, and column chromatographic separation of lipids. Importantly, Tom's group had synthetic phospholipids with single-species acyl chains, such as dihydrosterculoylglycerophosphorylcholine (DHPC), that resisted decomposition by heat or oxidation. Perfect!

I made many measurements on black films stabilized by DHPC. They were plagued by variations in specific capacitance and mechanical drift in the teflon aperture. I solved the mechanical drift problem (152) easily enough, but the measurements were still plagued by irreproducibility of the specific capacitance (165). One possibility was that I did not fully appreciate the boundary conditions of the films on the teflon septum, which led me to a detailed analysis of the shape of the torus of bulk material surrounding the black area based on the Euler equation (143). It was thus possible to describe accurately the shape of the torus surrounding the bilayer film. But

none of these advances solved the problem of time-dependent changes in specific capacitance. I was forced to conclude that the phase behavior of the torus, which is orders of magnitude more massive than the bilayer, must determine the properties of the bilayer film. Phospholipids are hygroscopic: As their moisture content varies, the phase behavior changes and affects the thickness and composition of the black film (152).

As my two-year Army tour of duty neared its end, Tom invited me to continue my black film work in his lab as a postdoc. I applied successfully for a National Institutes of Health (NIH) fellowship and moved to Charlottesville. In those days, one could also apply for support for postdoctoral training under the GI Bill. With support from both the NIH and the GI Bill, I was in a better financial position than I was as a beginning assistant professor!

CALIFORNIA

Because faculty positions were in short supply when I moved to Virginia, I very soon started applying for positions. It was discouraging. But one day, out of the blue, I received a phone call from Professor Peter Hall at University of California, Irvine (UCI). He had just accepted the chair of the physiology faculty in the new medical school and was recruiting faculty. Because of the reputation of the department at the University of Washington, he started his inquiries there, and my name was suggested. I visited the newly constructed UCI to give a seminar, and soon after was offered the first junior faculty position of the department. An important factor in my decision was that the University of California is one of the great academic institutions and was engaged in building a new campus next door to wealthy Newport Beach. It was destined to be successful. And, indeed, it has been.

I arrived at UCI in the spring of 1972. My startup funds amounted to $13,000! But that was sufficient, because Tom had let me take most of the lab equipment I had assembled. I knew, of course, that I was expected to win research grants to sustain the lab, so I applied for grants from both the National Science Foundation (NSF) and NIH. The rationale I gave to the funding agencies was that if we couldn't understand the interactions of simple alkanes with lipid bilayers, how could we possibly understand how proteins interact with lipid bilayers? NSF came through with an $8,000 grant. In those days, a beginning assistant professor would never receive funds to support a postdoc or graduate students on his or her grant; they were expected to be at the bench and to prove their worth.

BLACK LIPID FILMS

I continued the black film work using glycerol monoöleate (GMO) as the lipid because Denis Haydon had shown that equilibrium black films could be produced, providing that the aqueous phase was saturated with the slightly soluble lipid. I was thus able to carry out an extensive set of equilibrium measurements of black films using a wide range of alkanes and related hydrocarbons (144–148, 160). I also made extensive measurements of the temperature dependence of membrane thickness and bilayer composition that allowed me to define free energies of transfer between the bulk torus and the thin film (148, 149) (**Figure 3**). Based on these studies, I was promoted to Associate Professor with tenure in the spring of 1975. Unheard of today, almost all the crucial papers submitted for tenure were single-author ones. That apparently made NIH and NSF happy, because I was then able to expand my lab to include two postdocs.

ABANDONING BLACK FILMS

Although I learned much about the phase behavior and thermodynamics of alkanes in black films, two issues nagged me: the distribution of alkanes across the thickness of the bilayer and the

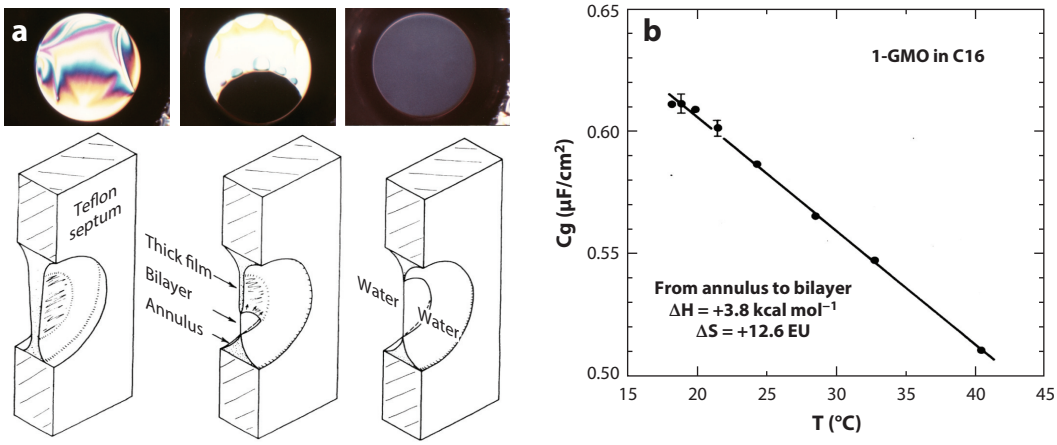


Figure 3

Work on "black" lipid membranes (BLMs). (*a*) Denis Haydon & Janet Taylor (39) and Paul Mueller and colleagues (93) introduced planar lipid bilayers. Dispersions of phospholipids in nonpolar solvents were spread across approximately 2-mm-diameter apertures drilled through polyethylene or teflon partitions. The films form spontaneously. The photographs in the upper panel show why the bilayer films are called BLMs. Electrodes placed in the aqueous compartments on either side of the BLM allow determination of the electrical properties of the film, especially specific capacitance C_m, which is inversely proportional to bilayer thickness (39, 160). The bilayers are surrounded by a torus, or annulus, of the bulk-forming solution (143, 152), allowing thermodynamic measurements of alkane partitioning between the bulk torus phase and bilayer phase to be determined (149). Panel adapted from Reference 164. (*b*) I made extensive measurements of the temperature dependence of the specific capacitance of bilayers formed from glycerol monoöleate and various alkyl liquids to determine free energies of transfer of alkyls between the bulk phase and bilayer phase. It was a happy day for me when *Nature* agreed to publish my measurements of the thermodynamics of transfer of hexadecane from torus to black film (148). As the chain length of the alkyl solvent increases, solubility in the bilayer decreases, as first noted by Denis Haydon and colleagues (5). For films formed using *n*-octadecane (C18), lowering the temperature below the C18 melting point caused loss of the C18 from the bilayer, leading to essentially solvent-free bilayers (146). This work led to the use of squalene, which is liquid at room temperature, as a solvent to form black films that are solvent free (150). Panel adapted from Reference 149. Abbreviations: C16, *n*-hexadecane; EU, entropy unit; GMO, glycerylmonoöleate.

conformation and dynamics of lipid acyl chains with respect to bilayer thickness. Thermodynamic measurements alone cannot answer those questions. To be successful in science, I decided, I must be prepared to abandon a beloved method and adopt appropriate new ones; I had to do whatever it took to answer pressing questions, even if it meant mastering new methods, such as x-ray or neutron diffraction. Because our knowledge of membrane protein structure was nil, I continued to pursue the notion that we should at least understand how small hydrocarbons interact with lipid bilayers. It was obvious that direct structural methods were absolutely required.

X-ray and NMR measurements of phospholipid–water mixtures had yielded fundamental information about their phase behavior and some basic structural information (89, 122). The most important advance for me was the 1971 foundational work of Levine & Wilkins (85) on the structure of oriented lipid bilayers. That paper, along with ones by Graham Shipley et al. (45); Anna Seelig & Joe Seelig (118); Georg Büldt, Joe Zaccai, and their colleagues (16); and Tom McIntosh, Glen King, and their coworkers colleagues (187), had established the tools and methods for describing the transbilayer profile structures of lipid bilayers. Building on the Levine & Wilkins work, David Worcester & Nick Franks (185) published an inspiring neutron lamellar-diffraction study of fluid egg lecithin–cholesterol multilayers in which they showed how specific deuteration made it possible to determine the transbilayer distribution of water and cholesterol in fluid-phase egg lecithin–cholesterol bilayers oriented on glass microscope slides.

NEUTRON DIFFRACTION WITH GLEN KING

I had heard a lecture by Benno Schoenborn at a meeting at Stanford about the neutron diffraction facilities at the High Flux Beam Reactor at Brookhaven National Labs (BNL). So I called him on the phone and arranged to visit him. Like so many other colleagues over the years, Benno was extremely generous with his time and resources. He invited me to undertake neutron experiments under the guidance of one of his instrument scientists, Jim Cain. The idea of the experiments was simple: use oriented arrays of fluid-phase dioleoylphosphatidylcholine (DOPC) bilayers at low hydration to determine transbilayer scattering-length density profiles of DOPC in the presence of protonated or deuterated *n*-hexane introduced through the vapor phase. In essence, subtracting the structure of DOPC bilayers with protonated hexane from the structure with deuterated hexane yielded the transbilayer distribution of the dissolved hexane (163). Jim Cain was unable to continue the experiments, but Benno told me that Glen King, then at the University of California, San Francisco, working on bacteriorhodopsin with Walter Stoeckenius, might be available to continue the measurements.

Glen joined the lab in 1980. He was extremely knowledgeable and experienced in diffraction methods and taught me the fine points. Besides being a smart, generous, and wonderfully kind human being, he was a first-rate mathematician. Glen traveled regularly to Brookhaven to collect neutron diffraction data on DOPC bilayers equilibrated with protonated or deuterated *n*-hexane. We published the results in *Nature* in 1981 (163) (**Figure 4**), showing that *n*-hexane

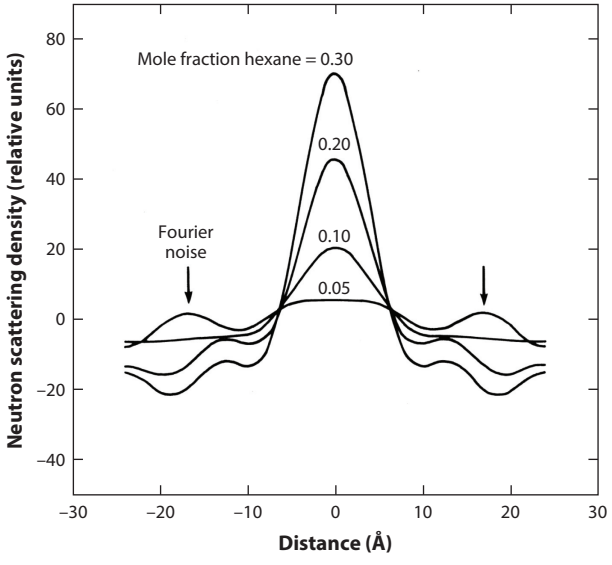


Figure 4

Locating *n*-hexane within lipid bilayers using neutron diffraction. Determining the thermodynamics of alkyl interactions with black lipid film bilayers was fun and useful, but thermodynamic measurements alone provide little structural information. With encouragement and help from Benno Schoenborn at Brookhaven National Laboratory, I pursued neutron diffraction structural measurements of dioleoylphosphatidylcholine bilayer stacks oriented on glass substrates. I was taught neutron diffraction, first by Jim Cane and then by Glen King. We varied the partial pressure of *n*-hexane inside the sealed aluminum sample chamber using *n*-hexane–*n*-hexadecane mixtures. This allowed us to establish that the dissolved hexane was located within the central 10 Å of the bilayer thickness (163). More important, neutron diffraction became an important structural tool for studying bilayer–protein interactions. This work precipitated nuclear magnetic resonance studies of *n*-hexane–phospholipid interactions (53, 54). Figure adapted from Reference 163.

was constrained to the central 10 Å of the bilayer mixed with the acyl chains, as hinted at by black film experiments (5, 15, 149, 151). Importantly, over several years, Glen painstakingly accumulated eight orders of neutron diffraction data on oriented DOPC multilayers at 66% relative humidity (RH) in the absence of hexane. The first four orders were easy, but the next four orders required huge amounts of data-collection time in addition to patience. One of Glen's gifts to the lab was eight orders of extremely high-quality neutron diffraction structure factors collected in the absence of *n*-hexane. I was deeply saddened when Glen passed away in 1992, the victim of a glioblastoma.

PEPTIDES WITH RUSS JACOBS

Continuing the theme of understanding the interactions of alkanes with bilayers, I also recruited Russell Jacobs to the lab in 1980. Russ is a terrific scientist with many talents. He earned his Ph.D. doing experimental and theoretical work on lipid bilayer systems with Hans Andersen and Bruce Hudson at Stanford, then did postdoctoral studies with Eric Oldfield at the University of Illinois at Urbana, where he constructed an NMR spectrometer for studying bilayer mixtures. Our collaboration started with a calorimetric and NMR study of hexane interactions with DMPC and DOPC bilayers (53, 54). An interesting conclusion was that acyl chain order determined by ^{2}H NMR was essentially unaffected by the presence of hexane, suggesting that the hexane was entrained by acyl chain motions. This was interesting, but it was time to abandon alkanes in favor of peptides.

Besides his extraordinary talents as a physical chemist, Russ is also excellent at chemical synthesis, which enabled the lab's transition to studies of peptide–bilayer interactions. Russ designed and synthesized a family of tripeptides of the form Ala-X-Ala-*O-tert*-butyl, X = Gly, Ala, Leu, Phe, and Trp. The peptides were sufficiently water soluble to allow physicochemical measurements of their interactions with DMPC and DOPC vesicles (55, 56). Importantly, the measurements yielded the lab's first peptide-based hydrophobicity scale. Russ also synthesized a version of the Trp peptide in which the Trp residue was deuterated. This allowed us to determine by neutron diffraction that the Trp residue was in the DOPC interface (57) (**Figure 5**). Funding for this work was problematic in the beginning. The NIH study section involved didn't like the work because we ". . .were not peptide chemists," despite having provided high-performance liquid chromatography (HPLC) and other data showing that we had synthesized the peptides successfully. The NSF was equally unenthusiastic. One project manager informed me that none other than Charles Tanford had told him that the experiments would not work. Nevertheless, the NSF provided six months of funding to prove the feasibility of the measurements, which we did.

RANDOM PROTEIN SEQUENCES

The importance of the hydrophobic effect (127) in protein folding and stability was well established by the 1970s. Starting with key papers by Joel Janin (58) and by Jack Kyte & Russell Doolittle (71), much effort was devoted to finding accurate hydrophobicity scales that would allow one to predict accurately TM helices using sliding-window averages with a window size of about 20 amino acids (28). There was much discussion about which hydrophobicity scale was best for predicting TM segments and amino acids buried in soluble proteins. The problem then, as now, was that not all hydrophobic residues are buried in soluble proteins, and charged residues are often found in TM segments. By 1987, there were more than 50 hydrophobicity scales (21)!

About the time that Russ was starting his own research group at UCI, we spent several afternoons discussing hydrophobicity scales and hydropathy plots and arguing about what the bumps and blips of the plots might be telling us about membrane protein structure. Then one day Russ

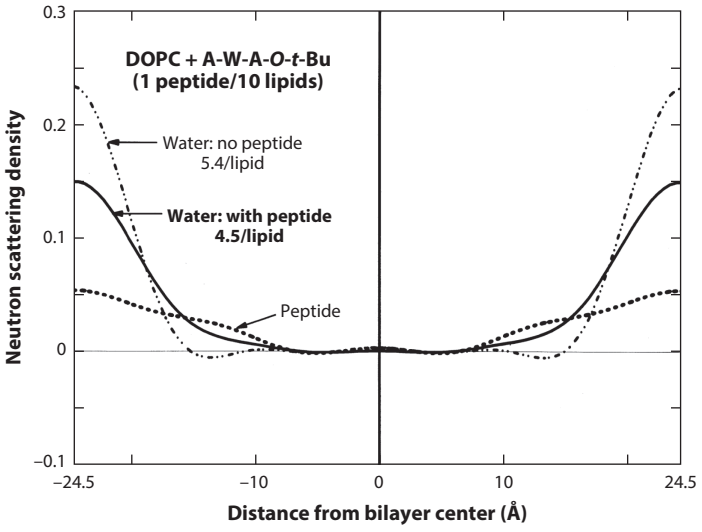


Figure 5

Locating peptides within lipid bilayers using neutron diffraction. Russ Jacobs started the lab down the path toward understanding, thermodynamically and structurally, the interactions of peptides with lipid bilayers. We began with a family of tripeptides of the form Ala-X-Ala-*O*-*tert*-butyl (A-X-A-O-*t*-Bu), which are readily soluble in the aqueous phase; the -*tert*-butyl group ensured bilayer partitioning into the bilayer interface (55, 56). We were particularly interested in the tryptophan-containing peptide due to the apparent interface enrichment of tryptophan in the structure of the photosynthetic reaction center determined by Deisenhofer et al. (25). We therefore determined by neutron diffraction the location of the Trp residue in oriented dioleoylphosphatidylcholine (DOPC) bilayer arrays using Trp-deuterated A-W-A-*O*-*t*-Bu peptide (57). This work was an important milestone in our journey toward gaining a full understanding of peptide–bilayer interactions. Figure adapted from Reference 57.

said, arguing that they meant little, "Maybe it's just noise." The best way to test that idea was to scramble the sequence of a membrane protein of known structure and have a look at the hydropathy plot. We scrambled the sequence of the L subunit of the photosynthetic reaction center over and over and ran hydrophobicity plots using a binary hydrophobicity scale (161); we always found five TM segments! This led to exhaustive studies of all of the approximately 5,000 sequences in the 1988 Protein Information Resource (PIR) database of protein sequences (35), which showed with remarkable accuracy that the PIR set of proteins could be described as a set of random sequences pretty much independent of which residues were designated as hydrophobic. This gave rise to the random origin hypothesis (153, 162), which had also been proposed by other labs using other approaches (84, 120). I was really excited by this, but the idea was pooh-poohed by all my friends and colleagues, especially crystallographers who could not believe that any aspect of their beautiful high-resolution structures might have any kind of random character. I abandoned the project. But recently, to my delight, Tretyachenko et al. (128) reported that "Random protein sequences can form defined secondary structures and are well-tolerated in vivo."

CLARIFYING THE FLUID LIPID BILAYER STRUCTURE PROBLEM

Glen King received his training in diffraction methods for partially ordered systems at the University of Michigan working with Roy Worthington. The problem that Glen had worked on with Allan Blaurock concerned the structure of nerve myelin, which can be thought of as a jelly roll of lipid bilayers (186, 187). In fact, the myelin lamellar diffraction studies from Worthington's lab

provided strong early evidence that the lipid bilayer was the basic structure of cell membranes. X-ray diffraction measurements obtained by swelling and shrinking the structure with sucrose solutions could provide up to about 12 diffraction orders. But with a *d*-spacing of about 400 Å, the resolution was at best about 30 Å (roughly the thickness of a bilayer). The standard practice in those days was to model myelin membranes using centro-symmetric strip-function models, which can be considered low-resolution images of bilayer profiles. As our attention shifted to diffraction studies of pure lipids organized as multilamellar arrays on glass or quartz substrates, the question was how to obtain useful bilayer structural parameters. Glen and I began to address this issue by asking how to extract from the strip model profiles the thickness of a bilayer's hydrocarbon core. We did this by first collecting eight orders of neutron diffraction data from DOPC multilayers oriented on quartz slides. The bilayer profiles obtained are shown in **Figure 6*a***.

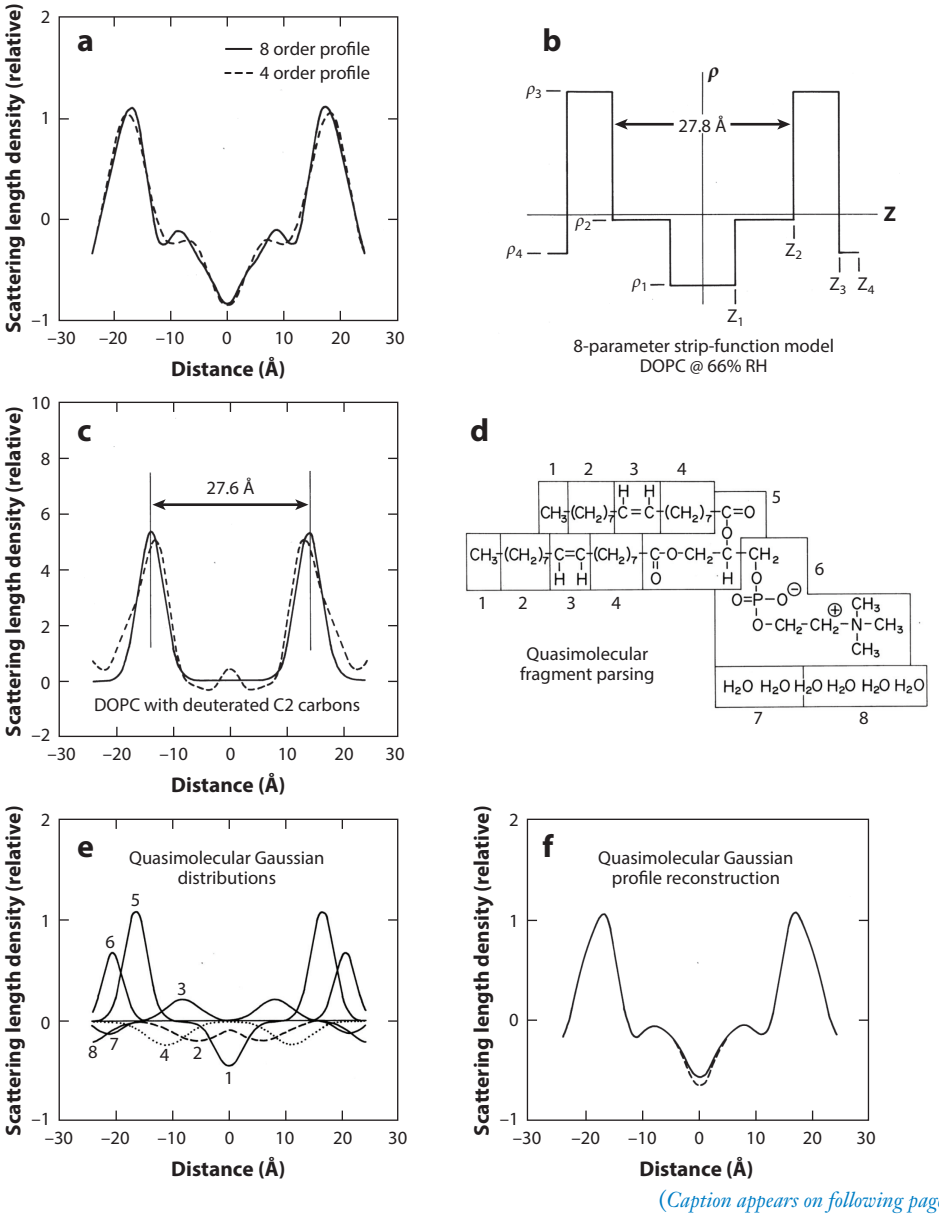
With eight orders of data, we could construct the strip model profile shown in **Figure 6*b*** using a fitting routine designed by Glen. The question was what bilayer structural parameters could be extracted accurately from the strip profiles. A simple interpretation was that $2Z_2$ corresponds to the thickness of the hydrocarbon core. We therefore obtained DOPC deuterated at the C2 carbon position. Subtracting the protonated profile from the deuterated profile yielded the transbilayer distribution of the C2 carbons (**Figure 6*c***). Remarkably, the transbilayer distance between the mean C2 positions of 27.6 Å agreed within experimental error with the $2Z_2$ value of 27.8 Å. In contrast to the hard edge of the strip model, the difference structure could be fit with reasonable accuracy with Gaussians that revealed the time-averaged transbilayer distribution of the C2 carbons. The width of the peaks revealed the thermal fluctuations of the bilayer. This led us to think more deeply about the problem: All of the structural components of the lipids must also be undergoing thermal fluctuations. In principle, with enough data, we should be able to determine the transbilayer distributions of the principal structural groups of DOPC. To test that idea, we broke DOPC into multiatomic "parts" consisting of lipid fragments (carbonyls, double bonds, phosphates, waters, etc.), which we called "quasimolecular" fragments (**Figure 6*d***). We represented each fragment as a transbilayer Gaussian function, the sum of which represents the transbilayer bilayer profile. The problem then became the insufficient number of measured parameters: Choosing to represent the bilayer profile as the sum of 8 Gaussians would require 16 parameters. A unique model could therefore not be determined. Nevertheless, with various "guesstimates" we determined approximate transbilayer Gaussians for the fragments (**Figure 6*e***), whose sum (**Figure 6*f***) yielded a decent profile similar to the experimentally determined profile (**Figure 6*a***) (66). This work set the lab on the road toward solving the transbilayer structure of fluid lipid bilayers using x-ray and neutron diffraction data (169–174).

SOLVING THE STRUCTURE OF A FLUID LIPID BILAYER

Glen took medical retirement shortly after the development of the quasimolecular modeling work, which was a tragic loss for me and the lab. The modeling effort was suspended, and I turned my attention to determining the transbilayer distribution of the Trp residue of Ala-Trp-Ala-*O-tert*-butyl in DOPC bilayers using neutron diffraction (57). One day, out of the blue, I received a phone call from Mike Wiener asking if he could do a postdoc in my lab. Mike was a student of John Nagle and had done beautiful physicochemical measurements on lipid dispersions, including x-ray diffraction measurements of dipalmitoylphosphatidylcholine (DPPC) dispersed in water.

Mike joined the lab in 1988 with the idea of continuing structural studies of lipid bilayers containing peptides. When he arrived, we discussed possible experiments. The idea had been slowly hatching in my brain that we might be able to determine the quasimolecular structure of fluid DOPC bilayers by combining, somehow, x-ray and neutron data. As a "warm-up" experiment, I

suggested that Mike carry out x-ray diffraction measurements on oriented DOPC bilayers under the same conditions as Glen's neutron diffraction measurements. I had become enamored with the idea of having two complete and independent DOPC diffraction data sets, one based on x-rays and the other on neutrons. This was possible because neutrons scatter from nuclei and x-rays from electrons, and the scattering lengths in the two cases are independent of each other. This means that there are 16 orders of diffraction rather than 8 for model building. Importantly, the two methods are sensitive to different bilayer features. For example, x-rays provide information on the headgroups that have high electron density, whereas neutrons are most sensitive to regions with low hydrogen content, such as the carbonyl groups.



(*Caption appears on following page*)

Figure 6 (*Figure appears on preceding page*)

First steps toward determining the structure of fluid lipid bilayers using joint refinement of x-ray and neutron data. What structural information can be extracted from bilayer profiles? That was the question that interested Glen and me. (*a*) Without further analysis of some kind, transbilayer profiles provide little information beyond the approximate locations of the phospholipid headgroups and the center of the hydrocarbon core. (*b*) One approach that was common at the time (around 1980) was strip models, from which we hoped to define more precisely the thickness of the hydrocarbon interior. Glen developed an algorithm for finding the best-fit strip model for our data. We wondered how the distance between the two prominent positive strips was related to the thickness of the hydrocarbon core. (*c*) To find out, we deuterated the C2 carbons. Remarkably, the peak-to-peak distance (27.6 Å) between the Gaussian peaks fitted to difference structure peaks agreed well with the distance (27.8 Å) between the prominent strip peaks. Importantly, the widths of the C2 carbon peaks revealed the thermal motion of the bilayer. We then realized that to describe the structure of the fluid bilayer, the principal structural (or component) groups would have to be represented by Gaussian distributions. (*d*) We divided the dioleoylphosphatidylcholine (DOPC) molecule into what we called "quasimolecular" fragments, (*e*) each of which were represented by a transbilayer Gaussian distribution (66). (*f*) Of course there were not sufficient data to make a unique model, but guesstimates about locations and widths of the Gaussians were encouraging. Figure adapted from Reference 66.

WATCHING THE NEUTRON COUNTS

The nub of the diffraction problem was why we could only observe at most eight orders of diffraction from DOPC bilayers oriented on flat glass slides. Suwalsky & Duk (126), for example, had reported 25 diffraction orders for stacks of oriented L-α-dimyristoylphosphatidylethanolamine (DMPE) bilayers on glass substrates at room temperature and low hydration. Of course, because our DOPC data were collected far above the phase transition temperature, we expected less ordered bilayer arrays and consequently fewer orders of diffraction. But what was the nature of our disorder? Following the definitive work of Hosemann & Bagchi (46) on disorder in crystalline systems, Kent Blasie's group had examined this question extensively for diffraction from ordered lamellar arrays of rod outer segments (117). Three types of disorder must be considered: thermal disorder, in which molecules fluctuate around well-defined positions within the unit cell; long-range disorder, which involves loss of spatial coherence (e.g., variations in the distance between bilayers in a stack of bilayers); and disorientation disorder due to the macroscopic geometry of a particular sample (e.g., different regions of the bilayer stack have slightly different orientations relative to the substrate).

I remember sitting at Benno Schoenborn's crystallography station at Brookhaven watching the intensities of the diffraction spots rise and fall as the glass slide with my oriented bilayer array was rotated in the neutron beam. The thing that struck me was that all the diffracted intensities (circular spots on the 2D detector) seemed to have the same width regardless of diffraction order (170). That meant that our stacks of bilayers had highly uniform spacing between bilayers; the disorder we observed was due only to thermal noise in the unit cell, as expected of a fluid bilayer. Mike made the same observation with his x-ray measurements. Thermal noise prevented us from identifying individual atoms in the layer, but we could observe groups of atoms, as in the quasimolecular models. After extensive "what if" modeling sessions, we developed what we called "composition space" refinement and showed that, with eight orders of neutron data and eight orders of x-ray data, we could generate accurate fluid bilayer models (171), provided that we could also determine independently the transbilayer distribution of the double bonds and water, which indeed we could (169, 172).

A difficult issue that we needed to resolve in our model building was the transbilayer distribution of the terminal methyl CH_3 groups of the lipids. Gruen & de Lacey (37), in early mean-field statistical mechanical models of fluid bilayers, found that the CH_3 groups likely extended into

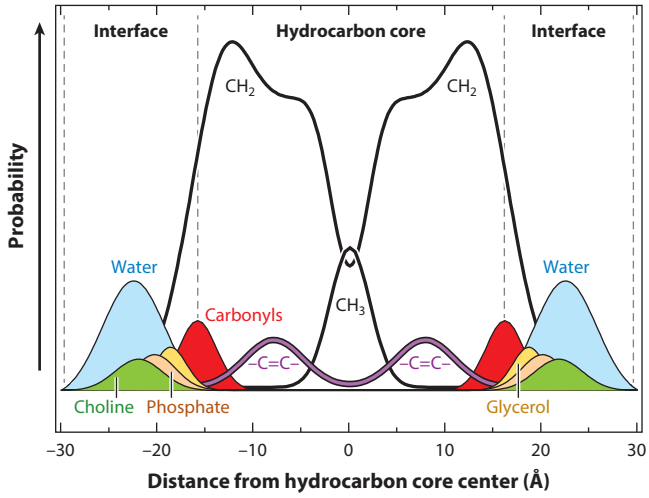


Figure 7

Bilayer structure determination by joint refinement of x-ray and neutron diffraction data. When Mike Wiener joined the lab in 1988, we began the development of the joint refinement method. The first step was to collect a high-quality set of x-ray data to complement Glen's neutron set. Mike collected all the x-ray data on DEF-5 photographic film using a 300-watt fixed-anode x-ray machine—the very same one that Alan Blaurock had used in his early x-ray studies of bacteriorhodopsin (10). Oldtimers will know that a stack of 8–12 films are used in such measurements and that analysis of the data requires performing an optical density scan of each film. The measured intensities of the peaks must account for the absorption of x-rays by the films. It was a huge amount of painstaking work (170) that set the stage for developing what we called composition-space refinement of x-ray and neutron data (171). Glen King had collected neutron diffraction data that Mike analyzed to determine the transbilayer of the double bonds and water (169). Mike used dioleoylphosphatidylcholine (DOPC) with brominated double bonds to determine the double bond distribution in x-ray space (172), which allowed us to determine the transbilayer distribution and packing of the terminal methyl groups (173). With these x-ray and neutron data in hand, we were able to determine by joint composition-space refinement the transbilayer structure of a fluid DOPC lipid bilayer (174), shown in this figure.

the headgroup region. Our data hinted that this might be true, but the experimental uncertainties restrained us from embracing the model. Consequently, we decided on modeling the distribution as a single Gaussian that was relatively narrow compared to the Gruen & de Lacy model (173). With this issue resolved in our minds, we were able to report the complete structure of the fluid DOPC bilayer in *Biophysical Journal* in 1992 (174), as determined by composition-space refinement (**Figure 7**). Since these measurements were reported, the general approach of joint refinement of x-ray and neutron scattering has been extended to a wide range of lipids and hydrations by John Katsaras, John Nagel, and their colleagues (70, 99).

EMBRACING MOLECULAR DYNAMICS SIMULATIONS

I reported our structure of the fluid DOPC bilayer at the 11th International Biophysics Conference held in Budapest in 1993. At the same meeting, Klaus Schulten described his 100 ps (!) all-atom molecular dynamics (MD) simulation of a palmitoyloleoylphosphatidylcholine (POPC) lipid bilayer (41). This was exciting! It was clear to me that our DOPC structure would be important for the validation of simulation methods. I made many visits to Klaus' lab to discuss the possibility and encourage a collaboration, but it never happened. I also participated in MD simulation workshops at the Biophysical Society and other meetings. I remember saying, "I won't believe

any of your simulation results unless you can simulate DOPC and obtain results that agree with our data." The key problem at the time was the lack of adequate MD force fields for unsaturated lipids. A particularly memorable meeting was one on membrane dynamics held at the University of North Carolina. All the pioneers of membrane MD attended, including Doug Tobias. He agreed with me that someone should focus on simulating DOPC under the same experimental conditions as in our experiments. Amazingly, one day in 1996 I received a call from the chair of our Chemistry Department asking if I would meet with Doug to help recruit him to UCI. Miracles do happen! Doug arrived at Irvine in 1997, and we quickly formed the TEMPO (Theory and Experiments in Membrane Protein Organization) group to bring together experimental and simulation types to work on membrane-related problems. At the top of the list, of course, was simulations of DOPC. Happily, by 1997, Scott Feller, Rich Pastor, and Alex MacKerell had developed force fields for unsaturated lipids and published the first DOPC simulation results (29), which generally agreed with our DOPC structure (174).

The central question for us was how to validate DOPC simulations rigorously using our data. Doug, his graduate student Ryan Benz, and my postdoc Francisco Castro-Román argued, correctly, that the best way was to run simulations of our DOPC bilayers and from them calculate the x-ray and neutron diffraction structure factors for direct comparison with the experimental structure factors (7). For the force fields then available, the structural parameters from the simulation agreed quite well with the experimental data using either GROMACS (8) or CHARMM27 (114). The one structural parameter that fell well outside experimental error in the simulations was the width of the CH_3 distribution (see **Figure 8**). Was the simulation in error, or was there a problem with the quasimolecular model? I was starting to feel certain that Mike's and my decision to represent the transbilayer CH_3 distribution as a relatively narrow Gaussian was wrong.

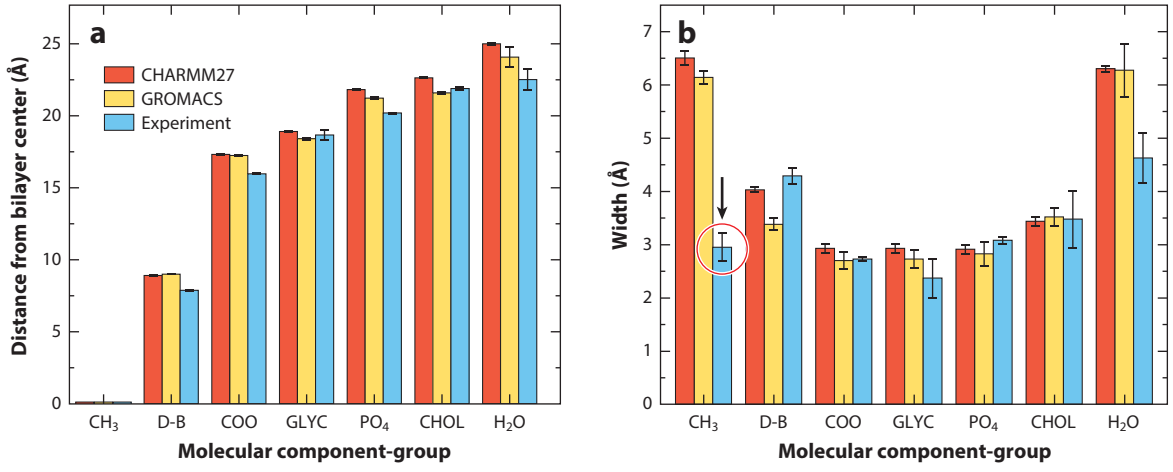


Figure 8

Testing the accuracy of molecular dynamics (MD) simulations. I had long wanted to find out if MD simulations of dioleoylphosphatidylcholine bilayers would agree with our structure. The arrival of Doug Tobias at University of California, Irvine, made this possible. The only rigorous way of doing the comparison was to compute neutron and x-ray structure factors for direct comparison with the experimentally determined structure factors. Doug's group did just that using both GROMACS and CHARMM27 force fields. The (*a*) transbilayer positions and (*b*) widths of the component groups determined in the simulations were compared with the joint-refinement experimental results. The simulation and experimental data were in excellent agreement except for a huge discrepancy in the width of the terminal methyl distribution (*arrow*). It was obviously important to establish the cause of the discrepancy, particularly because the MD simulations suggested deep penetration of the terminal methyl groups into the bilayer interface. Figure adapted from Reference 7.

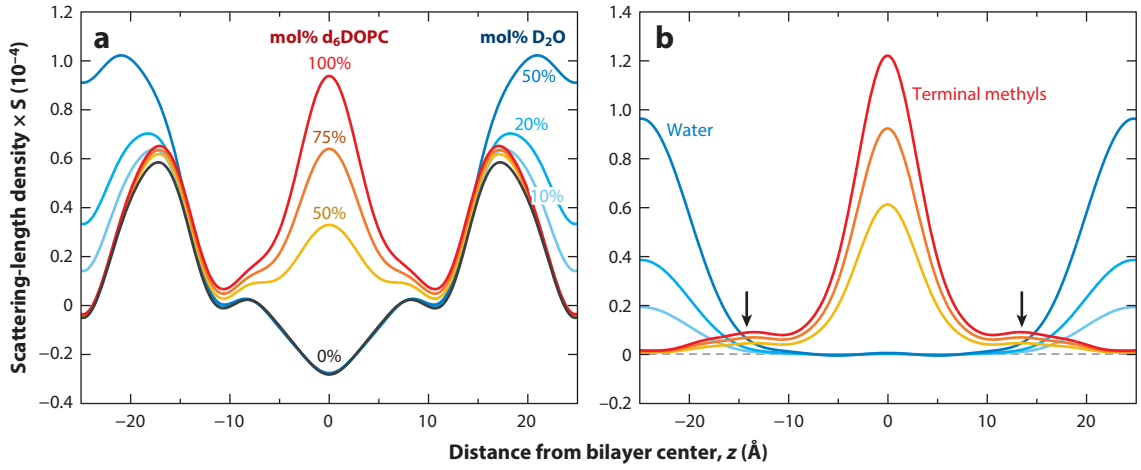


Figure 9

Experimental determination of the transbilayer distribution of the dioleoylphosphatidylcholine (DOPC) terminal methyl groups. To resolve the terminal methyl discrepancy, more experiments were essential. Our colleague Dick Chamberlin in the University of California, Irvine (UCI) Chemistry Department synthesized DOPC containing deuterated terminal methyls, which allowed us to determine accurately their transbilayer distribution. (*a*) Using the Advanced Neutron Diffractometer/Reflectometer the Cold Neutrons for Biology & Technology group had constructed at NIST (26), Ella Mihailescu and David Worcester performed exceedingly careful neutron diffraction experiments on bilayers with protonated or deuterated terminal methyls to obtain bilayer profiles under several conditions (92). (*b*) The difference structures reveal the deep penetration of the terminal methyls into the interface. The methyls spend about 10% of their time in the interface. These results confirmed the accuracy of the molecular dynamics simulations of DOPC. Figure adapted from Reference 92.

The only reasonable way to be sure would be a direct determination of the transbilayer distribution using specific deuteration. Dick Chamberlin in Doug's department synthesized DOPC with CD_3. Neutron diffraction experiments carried out at the NIST Center for Neutron Research using the Advanced Neutron Diffractometer/Reflectometer (AND/R) instrument by Ella Mihailescu and David Worcester, and simulations by Eduardo Jardón-Valadez and Francisco Castro-Román, showed that, indeed, the terminal CH_3 groups penetrated well into the headgroup region (**Figure 9**), thus validating the MD simulations (92).

COLD NEUTRONS FOR BIOLOGY AND TECHNOLOGY

Our early neutron measurements were performed at Brookhaven's High Flux Beam Reactor (HFBR). By the late 1990s, however, the HFBR encountered heavy political headwinds, and it ceased operation in 1999, leaving us with rather limited neutron capabilities. I had met several times, at various meetings, Jack Rush, who was then director of the NIST Center for Neutron Research (NCNR). NCNR operates a 30-MW reactor that produces a neutron beam cooled to liquid hydrogen temperatures (hence, cold neutrons). NCNR is a world-class facility dedicated to materials technology and fundamental science. Jack and I put our heads together and came up with a plan to create the Cold Neutrons for Biology & Technology (CNBT) program, supported jointly by NIST, NIH, and UCI. The goal was to design and construct the AND/R at the NCNR to support the needs of the membrane structural biology community (26). We organized a team of coinvestigators that included Charles Majkrzak, Susan Krueger, and Anne Plant at NIST; Doug Tobias at UCI; Huey Huang at Rice University; Kent Blasie at University of Pennsylvania; Tom McIntosh at Duke University; and John Nagle at Carnegie-Mellon University. The five-year grant

from the NIH for the CNBT was awarded in January 2001. Mathias Lösche (Johns Hopkins University and Carnegie-Mellon) was recruited as Director. David Worcester, one of the founders of membrane neutron diffraction, joined the team as a visiting scientist and eventually became a full member of the CNBT. The efforts of these outstanding scientists have assured the availability of a world-class facility for neutron diffraction/reflectivity studies on membrane systems.

MEANWHILE, PROGRESS ON THE PEPTIDE FRONT

Russ Jacobs' work had helped start the lab down the path of studying peptide–bilayer interactions. Just as Mike Wiener was wrapping up the bilayer structure work and preparing for a crystallography postdoc with Bob Stroud, Bill Wimley asked to join the lab following his Ph.D. with Tom Thompson. Bill is a terrific scientist of many talents. I readily agreed to have him join the lab, providing he could get an NIH postdoctoral fellowship. This was important because the lab was struggling financially.

Happily, Bill's postdoctoral grant application was approved. He arrived in April of 1991 to work on peptide-layer interactions in pursuit of a peptide-based hydrophobicity scale. The tripeptide work with Russ Jacobs had pointed the lab toward understanding the interaction of Trp with lipid bilayers, so Bill decided first to have a closer look at the interactions of Trp and Trp analogs with lipid bilayers (179–182) while he was figuring out an experimental framework for the hydrophobicity scale measurements. Importantly, Bill developed a method for measuring partition coefficients by HPLC (182), which was central to the development of our peptide-based hydrophobicity scales. The relevance of his Trp measurements increased dramatically when Schiffer et al. (113) reported that aromatic residues of membrane proteins tend to be enriched at the membrane interface, consistent with our neutron diffraction studies of Ala-W-Ala-*O-tert*-butyl at the DOPC bilayer interface (57).

As Bill's postdoc funds neared exhaustion, and with my ongoing funding challenges, we urgently needed to find a way to support him. To our rescue came Mike Selsted, an authority on antimicrobial peptides such as the defensins, who had been recruited to the UCI Pathology Department not long before Bill joined the lab. One of the standard tools for studying antibiotic peptides is to measure leakage of the contents of lipid vesicles induced by peptides. Bill was the perfect person to do such measurements. So, I basically rented Bill out to Mike. Bill worked in Mike's lab in the mornings and in my lab in the afternoon and evenings, using his time in my lab to design and synthesize peptides. A standard model for defensin-induced leakage was the formation of multimeric pores, which Bill investigated extensively (178). The collaboration with Mike saved the lab.

Bill worked on several peptide designs starting from the Ala-X-Ala-*O-tert*-butyl design. What we needed was a host-guest peptide that would partition without aggregation between water and bilayers and between water and octanol. After a lot of work on different designs, he noted in his meticulously maintained lab notebook, next to the Ac-WL-X-LL peptide, "May be acceptable." Despite continued work with the defensins in Selsted's lab, Bill synthesized and purified all 20 of the peptides. New funding finally arrived, which allowed Bill to focus primarily on measurements of peptide partitioning into lipid vesicles and *n*-octanol. The Ac-WL-X-LL peptides permitted us to determine a sidechain hydrophobicity scale relative to alanine, but we realized that we needed a whole-residue hydrophobicity scale that accounted for the cost of partitioning the peptide bond as well as the sidechains.

One day while we were discussing Bill's sidechain scale measurements as he worked at the HPLC purifying peptides, the answer became obvious: Use peptides of the form Ac-Trp-Leu_n. This system would yield the whole-residue Leu value, including the backbone. Because we knew

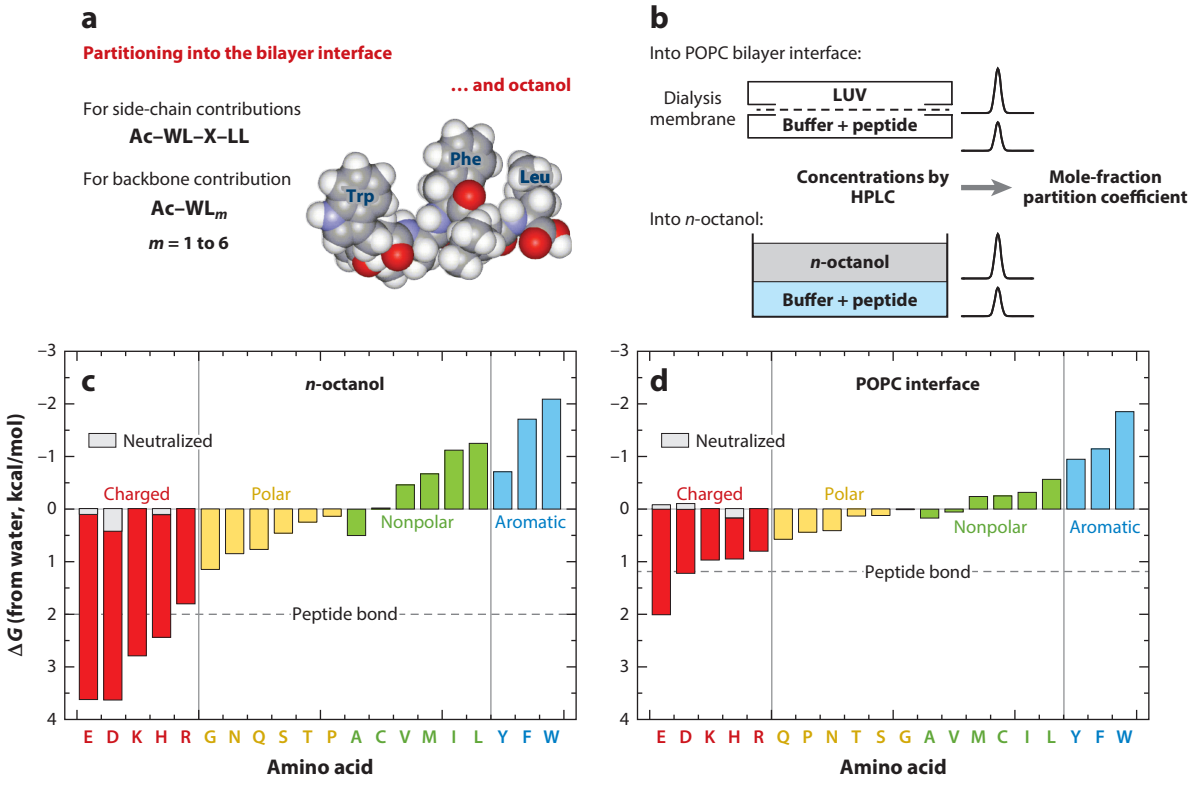


Figure 10

Whole-residue hydrophobicity scales. Until Bill Wimley's heroic measurements, most hydrophobicity scales [there were at least 50 of them (21)] were not derived from systematic experimental measurements. Furthermore, the existing scales did not account for the effect of neighboring residues. Missing entirely from the various scales was the energetic cost of partitioning the peptide bond. Bill's challenge was to find a family of hydrophobic peptides that did not aggregate in water or octanol. (*a*) The host-guest peptide system he developed, after a lot of failures, was Ac-WL-X-LL, where X is any of the 20 natural amino acids. The use of Leu residues was important because most membrane-active peptides and proteins are rich in Leu. Using the experimental scheme shown in panel *b*, Bill measured the partitioning of the peptides between octanol and water and between water and POPC large unilamellar vesicles. Including essential controls, the experiments required a lot of patience and several years to complete (175, 183). As is usual with this sort of approach, the sidechain hydrophobicities are determined relative to Gly or Ala. An important unanswered question was the cost of partitioning the peptide bond. We solved that problem using another family of peptides, Ac-WL$_m$, where m was varied from 1 to 6; knowing the free energy per residue allowed us to extract the peptide bond contribution. The results of Bill's measurements are summarized in panels *c* and *d*. Three features of these data are important. First, the cost of partitioning the peptide bond dominates partitioning into both *n*-octanol and the POPC interface. Second, a plot of the interface hydrophobicities against the octanol values is surprisingly linear with a slope of 0.5 (183). Third, the use of the whole-residue scales places hydropathy plots of protein sequences on an absolute scale that gives real thermodynamic meaning to the $\Delta G = 0$ reference line in the plots (61). Panels *c* and *d* adapted from Reference 155.

the sidechain value of Leu, we could then compute the backbone contribution and consequently whole-residue hydrophobicity scales (175, 183). We found that the energetic cost of partitioning the peptide bond into bilayers or octanol dominates partitioning free energy (**Figure 10**). For the octanol-to-water solvation free energy of transfer, we found -1.15 ± 0.11 kcal mol^{-1} for a glycyl unit. I was excited about this value, because John Edsall had reported -1.145 kcal mol^{-1} in his classic 1943 book with Edwin Cohn (20). In fact, I was so pleased that I wrote to John in 1996 noting that 53 years after his measurement, we arrived at the same value in an entirely different way. John replied, "Of course one does not expect, considering probable error, that the

agreement should be as near identity as it happens to be. Anyway, it is very pleasant to get such close agreement." Indeed! This is how experimental science should work.

A crucial contribution to this work was made by Trevor Creamer, a postdoc in George Rose's lab at Johns Hopkins. He carried out Monte Carlo simulations to determine the accessible sidechain areas of the peptides, which allowed us to calculate the hydrophobic solvation parameter σ introduced by Reynolds, Gilbert, and Tanford (103). We determined that $\sigma = 22.8 \pm 0.8$ cal $mol^{-1}Å^{-2}$ (175). This was a pleasing result and entirely consistent with the early estimate of Reynolds et al. of 21–25 cal $mol^{-1}Å^{-2}$ and an estimate of 22 obtained by Cyrus Chothia (19) based on amino acid solubility measurements of Nozaki & Tanford (96). The day we completed these calculations was a very happy one, especially for Bill, whose heroic measurements made it possible.

A useful feature of the Ac-WL-X-LL peptides was that the C terminus is charged. Trevor found from his simulations that $X = R^+$ or K^+ could readily interact with the COO^- terminus, which led us to collaborate with Klaus Gawrisch to determine by NMR the free energy of salt bridge formation in *n*-octanol (176). This was the first of several collegial and delightful collaborations with Klaus. A particularly important one was a close examination of the interaction of aromatic sidechains with the bilayer interface using NMR (189). We examined the interactions of several Trp analogs that Bill had examined earlier, such as 3-methylindole and indene, to expand our understanding of the interactions of AWA-*O-tert*-butyl with the DOPC interface (57). All analogs were found to be in the headgroup region near the C2 carbon. These observations ruled out the simple idea that the interfacial preference of aromatics was due to amphipathic or dipolar interactions. We concluded that the preference is likely "dominated by tryptophan's flat rigid shape that limits access to the hydrocarbon core and its π electronic structure and associated quadrupolar moment (aromaticity) that favor residing in the electrostatically complex interface environment" (189, p. 14713).

FRUITS OF COLLABORATION

Because of Bill Wimley's work on leakage from vesicles induced by human defensins, Mike Selsted suggested that our two labs collaborate further on mechanisms of action of antimicrobial peptides. Importantly, his lab had sufficient financial resources to recruit two new postdocs to work on defensins and a novel 13AA peptide, indolicidin, that Mike had discovered (133). I had met Alex Ladokhin and Kalina Hristova at the 1994 Biophysical Society meeting in New Orleans. Both were particularly interested in joining my lab. Kalina had done her graduate work on lipid vesicles made from polymer-grafted lipids with David Needham at Duke. As part of that work, she had also gained experience in x-ray diffraction methods with Tom McIntosh, also at Duke. She was a perfect fit for my collaboration with Mike Selsted on mechanisms of action of antimicrobial peptides.

Alex Ladokhin had received superb training in spectroscopy at the Institute of Biochemistry in the Ukrainian Academy of Science. He had come to the United States as a postdoctoral fellow in the laboratory of Peter Holloway at UVa and subsequently in the laboratory of Ludwig Brand at Johns Hopkins. He had done novel spectroscopic studies on the conformation of melittin, which was, and is, an important peptide for studying lipid–peptide interactions, as well as leakage of vesicle contents. He was another perfect fit for the Selsted–White collaboration. Among his many skills is a deep understanding of fluorescence spectroscopy that has been a key technique for studying peptide–bilayer interactions in our lab (74, 168).

Kalina and Alex are outstanding scientists whose interests and abilities extended well beyond leakage-of-contents studies using defensins and indolicidin. They joined the lab in late 1994, and both were anxious to participate in fundamental research on peptide–bilayer interactions using

studies of defensins and indolicidin as a springboard (48, 49, 76–78, 81, 82). Kalina was particularly interested in gaining more experience with x-ray diffraction and peptide–bilayer interactions. Her warm-up exercise was to extend Mike Wiener's structural studies of DOPC bilayers to include higher hydrations using x-ray diffraction measurements of DOPC bilayers with brominated double bonds. This led to a deep analysis of continuous transform phasing methods as well as fluid bilayer structures at higher water contents (50). The next step was to determine the location of α-helical peptides in lipid bilayers.

Jere Segrest and his colleagues Vinod Mishra and G.M. "Ananth" Anantharamiah at the University of Alabama had designed and studied extensively several amphipathic helical peptides as models for the Class A amphipathic helices of apolipoprotein A-I (119). Among the designs was one that they named Ac-18A-NH_2. To determine the location and orientation of the peptide in the bilayer and the nature of its perturbation of the bilayer, Kalina and I developed a new diffraction method, absolute-scale refinement, that relied on experimental determinations of absolute scattering-length density profiles along the bilayer normal and the transbilayer distribution of the brominated DOPC double bonds (52). Perfection of the absolute-scale method using Ac-18A-NH_2 allowed us to then determine the position of melittin in oriented lipid bilayers (47).

While we were developing these diffraction methods, Kalina also set to work to extend Bill Wimley's hydrophobicity scale measurements to include the water-to-bilayer transfer free energies of the various end groups commonly used in model peptides. These measurements provided a simple additive algorithm for predicting accurately the water-to-bilayer free energies of transfer of small peptides that lack secondary structure (51), including derivatives of the 13-residue antimicrobial peptide indolicidin that is notable for enrichment tryptophans and prolines and a lack of regular secondary structure (76). It was immensely satisfying that a plot of the experimentally determined free energies of transfer, plotted against predicted values, had a slope of 1.01 ± 0.01 and intercept of 0.06 ± 0.08, which is about as perfect as one can expect in an experiment.

A puzzling question about peptide partitioning into charged vesicles is the lack of simple additivity of electrostatic (ES) and hydrophobic (HΦ) effects in peptide partitioning into membranes. For example, the partitioning of melittin with 5 formal charges into membrane behaves as though it carries only 2.3 charges (the effective charge) (9). Alex addressed the additivity question using lipid vesicles formed from POPC–POPG mixtures, which permitted control of the membrane surface potentials. The hydrophobicity of indolicidins was controlled by synthesizing variants by replacing Trp with Leu, Phe, and Tyr in various combinations. He showed conclusively that the effective charge on the peptide depends strongly on the hydrophobicity of the peptide and provided a "rule of thumb" to account for the effect (80). The root cause remains to be determined.

WHY PEPTIDES ADOPT SECONDARY STRUCTURE UPON BILAYER PARTITIONING

Alex's extensive measurements of indolicidin interactions with lipid bilayers revealed that indolicidin partitions into bilayers without adopting regular secondary structure (75). This stands in strong contrast to the bilayer partitioning of the 26-residue bee venom melittin that forms α-helices upon binding to vesicles (136). Alex, an excellent optical spectroscopist, carried out extensive studies of the leakage of vesicle contents and developed, in collaboration with Bill Wimley, a fluorescence requenching method to distinguish graded leakage from all-or-none leakage of contents from large unilamellar vesicles (LUVs) (77, 81). Because melittin was known to cause vesicle leakage (100) and because of Alex's longstanding interest in melittin as a model peptide for studying lipid–protein interactions (73), it was natural for him to look more closely at melittin–bilayer interactions in my lab.

Melittin is largely unfolded in the absence of membranes but adopts an amphipathic helix when it partitions into membranes. Alex was naturally interested in the basis for this membrane-induced folding. If melittin didn't fold upon partitioning, then the free energy of transfer from water to bilayer would depend solely on its total hydrophobicity. To determine the energetics of melittin partitioning and folding, we needed a version that did not fold upon partitioning. In his studies of melittin-induced leakage vesicles, Yechiel Shai had synthesized a melittin D_4,L-diastereomer that did not fold upon partitioning (98). Yechiel generously sent us a sample of his diastereomeric melittin. Alex measured the partitioning free energy into vesicles of both versions of melittin. He found that wild-type partitioning was about 5 kcal mol^{-1} more favorable than the partitioning free energy of the diastereomer, and that the helicity difference between the two was about 12 residues. This meant that the helicity improved partitioning by about 0.4 kcal mol^{-1} per residue (79). Taking an entirely different approach using his $AcWL_6$ peptide, Bill found for β-sheet formation a value of about 0.5 kcal mol^{-1} per residue, but that number was uncertain due to uncertainties in the aggregate size of the interfacial peptide assemblies (177). These favorable partitioning values arise from the fact that it is less costly to partition hydrogen-bonded peptide bonds into the membrane interface than free peptide bonds. A collaboration with Paulo Almeida (1) using data collected by Mónica Fernández-Vidal and other members of my lab (31) found that the refined free energy reduction per residue value is -0.37 ± 0.02 kcal mol^{-1} per residue, similar to the value first determined by Alex.

An interesting question about melittin is its conformational dynamics when partitioned into membranes. Is there a wide range of states that, on average, give the observed CD signal, or is the range very narrow? Magnus Andersson answered this question in collaboration with Martin and Jakob Ulmschneider using microsecond-scale simulations of melittin on DOPC bilayers: It's a narrow distribution (4). Magnus, a student of Richard Neutze and Jan Davidson at Chalmers University of Technology in Göteborg, had developed a time-resolved x-ray method for visualizing the dynamics of halorhodopsin using heavy atom substitutions. Furthermore, he had used MD simulations to predict the outcome of the time-resolved x-ray scattering experiments. He contacted me because he was going to be in Los Angeles and wanted to visit the lab to talk about doing a postdoc with me. He visited, I offered him a position, and he joined the lab in 2009. Magnus was another of my "out of the blue" postdocs. I rarely have had to recruit postdocs; they just seem to appear at the right time. Like Mike Wiener, Alex Ladokhin, and Kalina Hristova, they have simply been interested in the lab's research and had skills and training that fit naturally with the lab's approaches to membrane biophysics.

COMMITTING IDEAS TO SOFTWARE

Sajith Jayasinghe, a Ph.D. student of David Cafiso at UVa, became interested in the lab after hearing me speak at a 1996 symposium honoring Tom Thompson upon his retirement. When Sajith joined the lab in 1999 after finishing his NMR studies with David (60), we decided that it would be useful to develop software based on the lab's peptide partitioning data. A problem of particular interest was the prediction of TM helices in membrane proteins and the usefulness of our hydrophobicity scales in that regard. As a first step, Sajith and Kalina carefully curated a database of membrane proteins of known structure that provided accurate information about the locations of the TM helices in the amino acid sequences. To make the data accessible, they developed the MPtopo database (62).

An important question was the prediction accuracy of popular hydrophobicity scales, including the two scales that Bill Wimley had determined. The crucial issue was that none of the commonly used scales accounted for the partitioning of H-bonded peptide bonds, which we had determined

to be 1.15 kcal mol^{-1}. Sajith developed an algorithm for assessing the prediction accuracy of hydrophobicity scales. It turned out that the "Wimley-White" scale was the most accurate, but the accuracy of the other scales examined could be improved dramatically by accounting for the energetic cost of partitioning the neglected H-bonded peptide bonds (6, 57, 61, 157). These results confirmed an earlier analysis by Nir Ben-Tal, Barry Honig, and colleagues that indicated the importance of the peptide bond in TM helix stability (6). Sajith and Kalina's next move was to embed our understanding of the energetics peptide-bilayer interactions in a software tool, Membrane Protein Explorer (MPEx) (**https://blanco.biomol.uci.edu/mpex**). The code for the software was developed by Craig Snider (see 124).

When Bill Wimley and I wrote a review on lipid–protein interactions for Annual Reviews (167), we listed all 12 (!) membrane proteins of known 3D structure. I thought it would be useful to create a website to keep track of the progress of the determination of membrane protein structures, along with their Protein Data Bank codes and literature references. So I created a hand-edited HTML-based mpstruc web page (**https://blanco.biomol.uci.edu/mpstruc**). It was easy to maintain in the beginning because new structures appeared relatively slowly; there were only 75 unique structures by 2003 (156)—15 years after the determination of the structure of the photosynthetic reaction center (25). As the number of new structures grew exponentially, however, manual maintenance of the HTML code for mpstruc soon got out of hand. Craig to the rescue! He created an SQL database and software to simplify maintenance. At this writing, there are more than 1,500 unique structures.

"LOUIS, I THINK THIS IS THE BEGINNING OF A BEAUTIFUL FRIENDSHIP"

Humphrey Bogart's closing line in *Casablanca* always reminds me of Gunnar von Heijne. We had been invited to a meeting on membranes in Urbana in 2002: Gunnar to talk about membrane biogenesis and I about lipid–protein interactions. Walking back to our hotel after the meeting banquet, I told him that I would love to move the lab's research toward more realistic biological systems like he was working on (111) to test our hydrophobicity scales. But I told him that, alas, it would take years to convince the NIH to provide the funds for such a drastic move by a physics type. Gunnar immediately suggested that we collaborate (139), and I traveled to Stockholm to discuss the experiments. The main idea that materialized was to use two-helix *Escherichia coli* signal peptidase construct with an added helix (H-segment) of the form GGPG-X-GPGG with X = 19 amino acids of our choice (**Figure 11*a***). If the H-segment was greasy enough, then it would be inserted as a TM segment; otherwise, it would be secreted across the membrane. This was, indeed, the beginning of a beautiful friendship!

Tara Hessa, a graduate student in his lab, spearheaded the effort. Hundreds of constructs later, we reported our "biological" hydrophobicity scale in *Nature* (42) (**Figure 11*b***). *Nature* initially rejected the work, but fortunately, *Nature* loves the scientific opinions of Nobel Laureates. They asked Rod MacKinnon—who had just won the Chemistry Prize for his work on ion channels—to bring to their attention papers worthy of publication in *Nature*. Rod said, "Well, you just rejected one!" Suddenly, *Nature* was interested in our first biological hydrophobicity scale paper and subsequently the second one (43).

The biological scale describes the apparent selection rules that translocons follow in selecting H-segments for retention in the membrane. But it is a black box: Knowing the rules provides no information on mechanism. The assumption is generally made that nascent amino acid chains pass through the translocon and, if sufficiently hydrophobic, partition between the translocon and the lipid bilayer. That must be true in a broad sense, but the situation is more complex, and

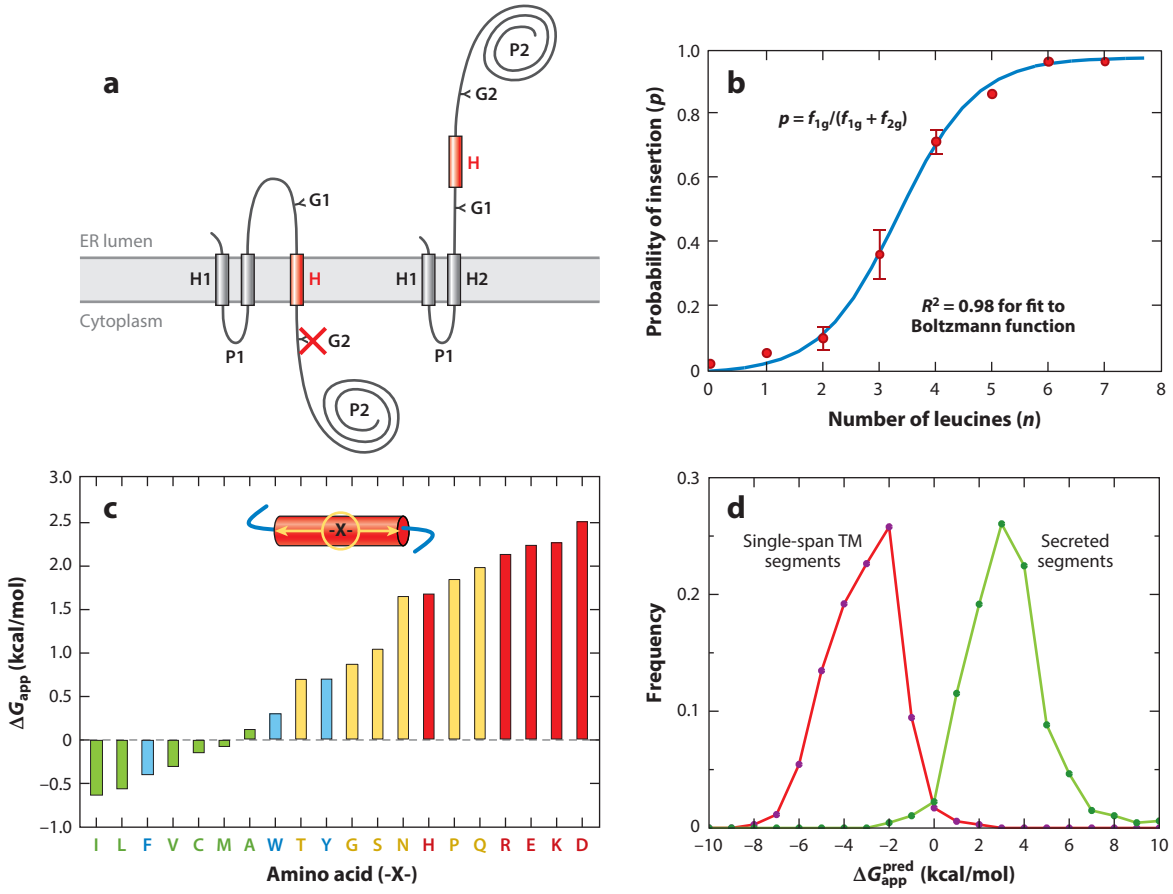


Figure 11

Biological hydrophobicity scales. Gunnar began pioneering work in the late 1980s on the determinants of membrane protein topology using *Escherichia coli* leader peptidase (Lep) that has two TM segments (3, 138). In the late 1990s, he switched to expressing Lep in a mammalian microsomal system and introduced glycosylation mapping for determining TM helix topology (95). (*a*) The approach involves inserting a third potential TM helix (H-segment) into the P2 C-terminal domain by site-directed mutagenesis. Because glycosylation occurs only in the microsome lumen, protein topology can be investigated via strategic insertion of Asn-X-(Thr/Ser) glycosylation sites in the sequence. About the time that Gunnar and I began our collaboration, Tara Hessa, Magnus Monné, and Gunnar had perfected the system shown. This set the stage for our "biological" hydrophobicity scale measurements using designed H-segments of the form GGPG-H-GPGG, where H is a designed 19AA sequence consisting of symmetrically arranged Ala and Leu residues with the X residue in the center of the segment (42). Glycosylation sites at each end of the H-segment allowed us to quantitate the probability of TM insertion from $p = f_{1g}/(f_{1g} + f_{2g})$; inserted segments were singly glycosylated and secreted segments were doubly glycosylated. Our first measurements began with an H-segment of 19 Ala residues, which was almost entirely secreted. (*b*) We then made a series of Ala→Leu substitutions, which showed that the probability of insertion followed a Boltzmann distribution. In subsequent experiments for the remaining 19 natural amino acids, we made as many Ala→Leu substitutions as necessary to keep $p \approx 0.5$ to assure maximum sensitivity of the assay. The apparent free energies of insertion were calculated from $\Delta G^{X}_{app} = -RT \ln K_{app}$, where $K_{app} = f_{1g}/f_{2g}$. The resulting biological scale is shown in panel *c*. (*d*) The next step was to determine the position dependence of the apparent free energy of X within a TM segment (43). This led to an algorithm for predicting the apparent free energy of insertion ΔG^{pred}_{app} of any amino acid sequence. Running the algorithm on a collection of single-span TMs and a collection of secreted proteins cleanly separated the two classes. Panels *a–c* adapted from Reference 42. Panel *d* adapted from Reference 43. Abbreviations: ER, endoplasmic reticulum; TM, transmembrane.

more interesting, than the standard cartoon-like models suggest (23, 116, 159). Understanding the process at the molecular level has occupied the lab since the Hessa et al. papers appeared.

A GREAT TEAM

The beauty of the collaboration with Gunnar is that my lab does physical chemistry measurements that complement the in vivo work in his lab. Because of the UCI TEMPO group, it was natural to draw Doug Tobias' lab into the collaboration to do related MD simulations. One of the first things we did was examine the effect of helix length on insertion (59). As expected, the shorter the all-Leu H-segment, the lower the probability of insertion. Mónica Fernández-Vidal synthesized a family of GGPG-L_n-GPGG (n = 6–12) and determined their dispositions in oriented bilayer arrays using circular dichroism. At the same time, Simon Jaud from Doug's lab carried out extensive MD simulations that showed that the energetics of insertion was strongly influenced by hydrophobic mismatch—the shorter the helix, the greater the energetic cost.

Mónica Fernández-Vidal, another of my "out of the blue" postdocs, completed her Ph.D. in 2002 at the University of Graz with Peter Laggner using x-ray diffraction to study the effect of salts, cholesterol, and melittin on phospholipid membranes. Because she wanted to expand her expertise in peptide–bilayer interactions in my lab, she wrote to ask about doing a postdoc. I was going to a meeting in Paris and invited her to meet me there for an interview, which went very well. She joined the lab in 2003, worked closely with Alex, and made crucial contributions to our understanding of peptide–bilayer interactions (18, 30–32, 59, 72).

REALLY? ARGININE IN MEMBRANES?

Rod MacKinnon proposed in his first structure of a voltage-gated potassium channel (63, 64) that the Arg-rich S4 helix of the channel, buried in the lipid bilayer, moved in the membrane electric field to cause channel opening. There was a lot of pushback, because surely a TM helix carrying four Arg could not possibly be buried in the membrane. Tara Hessa, Gunnar, and I could test that challenge by simply using the KvAP S4 segment in our in vivo expression system (44). We found that the insertion ΔG for the segment was about zero. This meant that the S4 helix was inserted into the membrane about 50% of the time, which is far more favorable than the critics believed possible. Alfredo Freites from Doug's lab carried out extensive MD simulations of S4 in a lipid membrane, which told us how this could happen (33): Arg snorkeling to the interface (65) forms a hydrogen-bonded network of water and lipid phosphates around the Arg residues, accompanied by local bilayer thickness reductions.

But what if the lipid used did not have phosphate groups? Rod MacKinnon's lab discovered that negatively charged phospholipid molecules were essential for the function of the KvAP channel (115). To understand why, Magnus Andersson carried out extensive simulations of the S4 voltage sensor in dioleoyltrimethylammoniumpropane, which is essentially DOPC without a phosphate group. His simulations showed that, in bilayers lacking lipid phosphates, the charged Arg residues of S4 are solvated by chloride counterions, water molecules, and the carbonyl groups of the surrounding lipids. In the resulting protein–lipid interface, the Arg residues lack the necessary phosphate anchor points to the lipid bilayer.

This work set the stage for a structural study of the S1-S4 voltage-sensing domain embedded in oriented lipid bilayer stacks. The samples were suitable for NMR measurements in Klaus Gawrisch's lab at NIH and neutron diffraction measurements at NIST using the AND/R (67). It was a great team effort between my lab (Mihaela Mihailescu and David Worcester) and the labs of Kenton Swartz (Dmitriy Krepkiy) and Doug Tobias (Eric Schow and Alfredo Freites). Our studies showed, as suggested earlier by Rod (see 115), that voltage sensors have evolved to interact with

the lipid membrane while keeping energetic and structural perturbations to a minimum, and that water penetrates the membrane to hydrate charged residues and shape the TM electric field.

HOW DO TRANSLOCONS ACTUALLY WORK?

The first high-resolution structure of a translocon (101, 135) and earlier work from Tom Rapoport's lab (40) suggested that all membrane proteins that emerge from the ribosome pass through the translocon. When a sufficiently greasy segment appears, it partitions into the lipid bilayer from the translocon via the so-called lateral gate. While this scheme has always been broadly accepted, the insertion of the S4 segment (44) and our determination of the hydrophobicity of Arg and Lys residues caused consternation among physical chemists and MD simulation people. Skipping over the gory details, MD simulations estimate that the cost of inserting an Arg residue into the membrane is at least an order of magnitude larger than observed in the Hessa et al. experiments. We examined this issue carefully to resolve the problem (116) and concluded, as in our earlier simulation of the S4 helix, that snorkeling of the Arg to the interface greatly reduces the free-energy cost. We also noted that the translocon is not dealing with a single Arg, as it does in the simulations, but with a 19-residue H-segment that contains six Leu residues as well.

These and other considerations led us to a broader consideration of the problem (116) (**Figure 12**). The most fundamental issue is that the Hessa et al. experiment measures the free energy of transfer from the translocon to the bilayer (ΔG_{app}), whereas the MD simulations

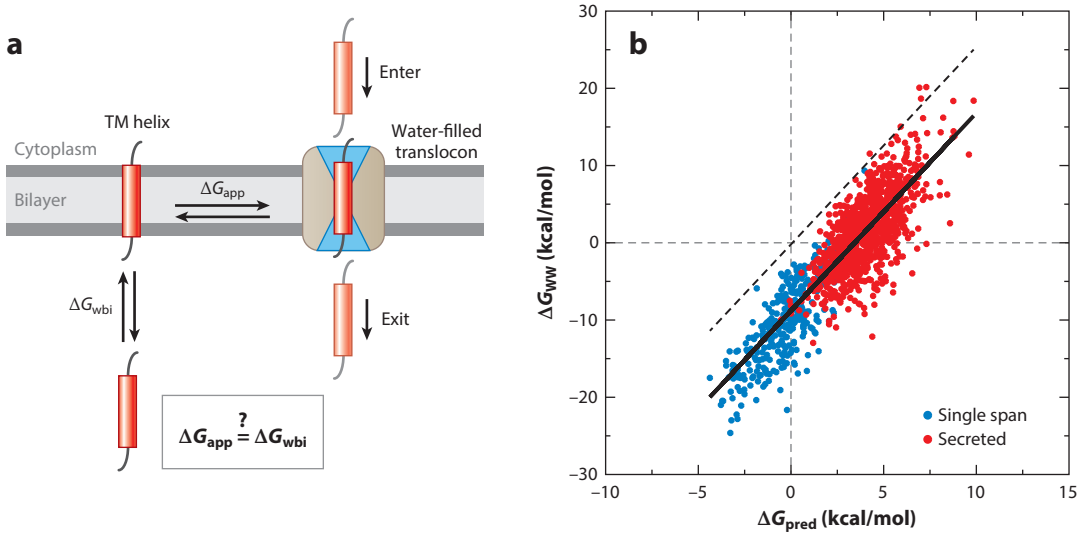


Figure 12

The connection between physical partitioning of transmembrane (TM) helices and translocon-assisted partitioning. My, Doug's, and Gunnar's labs spent considerable time on this question (116). (*a*) If the translocon as gateway into the bilayer is little more than a pore filled with bulk water, then one could reasonably assume $\Delta G_{app} = \Delta G_{wbi}$. (*b*) One step we took, using the same protein segments as those in **Figure 11*d***, was to compare the prediction of the Wimley-White octanol scale ΔG_{WW} to ΔG_{pred}. The scales were linearly related by a simple relation: $\Delta G_{WW} = -8.79 + 2.56\Delta G_{pred}$. Were it not for the offset of 8.79 kcal mol^{-1}, would both scales distinguish equally single-span TM segments from secreted segments? We discussed the meaning of these numbers extensively (see 116). One possibility was that the solvation parameter σ for water in the translocon was 8.8 cal mol^{-1} Å^{-2}, rather than the 23 cal mol^{-1} Å^{-2} found for bulk phase partitioning. We noted "...the possible usefulness of a solvation parameter for describing the polarity of the translocon" (116, p. 46). One way to determine this was to initiate molecular dynamics simulations of translocons to understand the behavior the 400 or so waters within the translocon. Figure adapted from Reference 116.

estimate the free energy transfer from water to bilayer, ΔG_{wbi}. So, the real question is whether $\Delta G_{app} = \Delta G_{wbi}$. As a first step, using a collection of single-span membrane proteins and a collection of nonspanning helices (43), we compared the insertion free-energy cost ΔG_{WW} calculated using the Wimley-White scale to ΔG_{pred} for the translocon scale using the algorithm of Hessa et al. (43). We ultimately concluded that the discrepancy between the two scales could be explained if the solvation parameter σ for the free energy of transfer of the H-segment from the "water-filled" translocon to the bilayer hydrocarbon core was about 9 cal mol^{-1} $Å^{-2}$, rather than the 23 cal mol^{-1} $Å^{-2}$ expected for bulk-phase transfer determined by Wimley, Creamer, and White (175). This implied in simple terms that the water inside the translocon was not equivalent to bulk water.

Hiroaki Suga at the University of Tokyo is a master of a suppressor transfer RNA–based technique to introduce nonproteinogenic amino acids into proteins. In collaboration with him, it was possible to introduce aliphatic and aromatic amino acids into our H-segments (97). For example, we could determine ΔG_{app} for a series of aliphatic sidechains of increasing length. We estimated from these measurements that the solvation parameter varied systematically along the length of the H-segment from about 6 cal mol^{-1} $Å^{-2}$ near the surface of the membrane to 10 cal mol^{-1} $Å^{-2}$ at the apparent translocon center, entirely consistent with the back-of-the-envelope estimate of Schow et al. (116). A titillating result, to be sure!

SIMULATING TRANSLOCONS

I received an email from Nicoletta Bondar in the spring of 2005 saying that she would be traveling in the United States and wondering if she could stop by to discuss doing a postdoc. Of course, I said yes. Nicoletta had extraordinary credentials and was working with Jeremy Smith in Heidelberg on proton transfer in bacteriorhodopsin. She joined the lab in November of 2006 to begin work on MD simulations of the translocon. Nicoletta, I soon learned, is a master of how H-bond networks control protein dynamics (13). She applied her skills and insights to the *Methanococcus jannaschii* SecYEβ translocon and mapped the remarkable H-bond networks (11) (**Figure 13**). These networks give the translocon structural stability and, at the same time, the flexibility necessary to respond to *all* protein segments presented by the ribosome. Not too long after she came to the lab, three different groups, almost simultaneously, reported the structures of the rhomboid intramembrane protease (GlpG in *E. coli*) (158). We were intrigued by this structure because of its symmetry with the translocon: Single-span membrane proteins must *enter* GlpG in the plane of the membrane to be cleaved, whereas single-span proteins *leave* the translocon in the plane of the membrane. Nicoletta carried out a definitive simulation of GlpG that revealed how its H-bond network participated in function (12). A particularly significant result was how GlpG distorts the membrane locally. This was later shown to be extremely important in the diffusion of the protein in the plane of the membrane (68).

Sara Capponi joined the lab in 2011. An excellent young physicist, she had extensive training in neutron scattering from polymer solutions, but little experience with proteins. She came to the lab to take part in the neutron diffraction studies of ion channels, but with a temporary shutdown of the NIST reactor, that plan was sunk. We decided it would be good for her to work with Nicoletta to learn about proteins at an intimate level. I also sent her to several workshops to learn more biophysics. We were intrigued by a SecYEβ translocon structure published by Egea & Stroud (27) that revealed a translocon with a partially open lateral gate. One immediate question was whether the translocon gate would close under prolonged simulation. We determined that it didn't, but the more important issue was the behavior of the water inside the translocon. Was it simply a pore filled with bulk water? The answer was no (**Figure 14**). Sara found that the water molecules inside

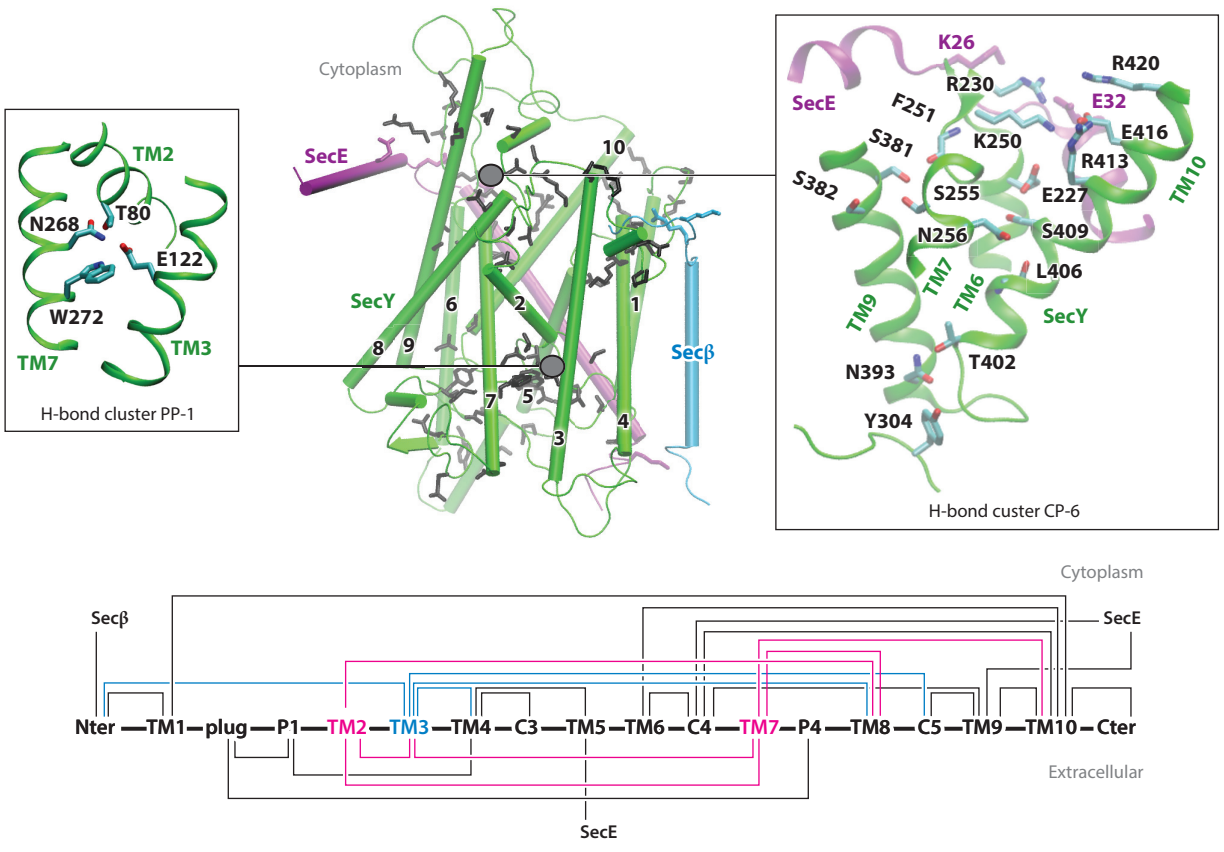


Figure 13

An extensive hydrogen bond network stabilizes the SecY translocon in membranes. Nicoletta Bondar initiated our first translocon simulations (11) using SecYEβ from *Methanococcus jannaschii*, whose structure had been determined by van den Berg et al. (134). The intraprotein hydrogen bond network discovered by Nicoletta was remarkable. It implies a highly stable, but flexible, protein that can respond to whatever demands the bilayer, nascent protein chains, and ribosome place on it. Figure adapted from Reference 11.

the translocon behaved anomalously; they were highly oriented with slow relaxation times near the translocon midplane but behaved more bulk-like as the membrane surface was approached (17).

Bill Wimley and I had compared the partitioning free energies for the pentapeptides into the membrane interface with the free energies for bulk-phase partitioning into octanol (183). The two scales were highly correlated with a slope of 0.5, suggesting that the solvation parameter for the interface was 11–12 cal mol^{-1} $Å^{-2}$, similar to the value obtained for the translocon by Öjemalm et al. (97). Together, these data suggested that the solvation parameter was related to water relaxation times. This raised the question of what the relaxation times of waters in the membrane interface are. Sara and a former graduate student in the department, Venki Krishnamani, found that water relaxation times in the bilayer interface were indeed similar to those observed in the translocon (69). Recently, Karen Fleming and her student Dagan Marx reported that the "translocon energetically mimics the bilayer interface" (91, p. 764).

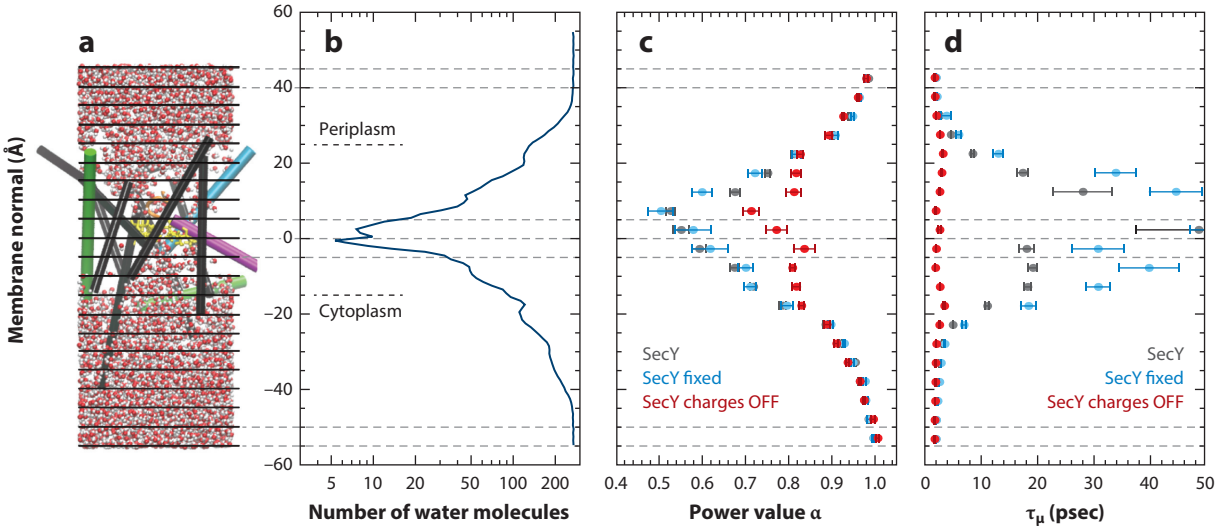


Figure 14

Water within the translocon does not behave like bulk water. Egea & Stroud (27) published a structure of the *Pyrococcus furiosus* translocon whose unusual feature was that the lateral gate was more open to the lipid bilayer than in previously published structures. This "primed" state arose because the long C-terminal helix of SecY acted as a substrate mimic during crystallization. We wondered whether the translocon would close in simulations of SecY in the absence of the mimic. While Sara Capponi was working on this simulation, the measurements of Öjemalm and colleagues (97) revealed that the hydrophobic solvation parameter σ was about 10 cal mol^{-1} $Å^{-2}$ rather than the canonical 23 cal mol^{-1} $Å^{-2}$. (*a*,*b*) Consequently, Sara turned her attention to the properties of the approximately 400 water molecules within the translocon. Those waters, she found, behaved anomalously: (*c*) They exhibited anomalous diffusion (so-called subdiffusion, indicated by $\alpha < 1$), (*d*) had highly retarded rotational dynamics, and aligned their dipoles along the SecY transmembrane axis. The translocon is therefore not a simple water-filled pore, entirely consistent with our earlier hypothesis that the solvation parameter within the translocon might be smaller than in bulk water (**Figure 12**). By the way, the translocon remained stably open throughout Sara's simulation. Figure adapted from Reference 17.

SIMULATING TRANSMEMBRANE HELIX INSERTION

About the same time that we published the biological hydrophobicity scale paper (42), Martin Ulmschneider—who had done his Ph.D. with Mark Sansom at Oxford—and his colleagues published a statistical analysis of the transmembrane preferences of amino acids for a collection of high-resolution membrane protein structures (131). For example, aromatic residues have a strong preference for the membrane interface. We were excited that the preferences Martin determined mirrored the preferences we had determined: Translocon preferences recapitulate membrane protein preferences! One could see how the translocon-assisted assembly process was setting up the proteins to fold into their native states. In 2008, I unexpectedly received a message from Martin expressing interest in coming to the lab under a Marie Curie Outgoing Fellowship. I was delighted! He and his twin brother Jakob were doing a lot of fundamental work on force fields and simulations of TM helix insertion into bilayers. Martin's interests perfectly matched mine. After a huge "Eurocration" over the Marie Curie Action, Martin arrived in the lab for a two-year stay in 2009. An added value of having Martin in the lab was that Jakob would show up from time to time to discuss science.

Martin and Jakob had already started running μsec simulations of the folding into bilayers of polyleucine H-segments L_n with *n* ranging from 4 to 12. Importantly, they were run until equilibrium was reached. It was a beautiful experiment. The free energies of insertion are easily calculated at equilibrium from the fraction of time the peptides spent on the membrane surface versus the

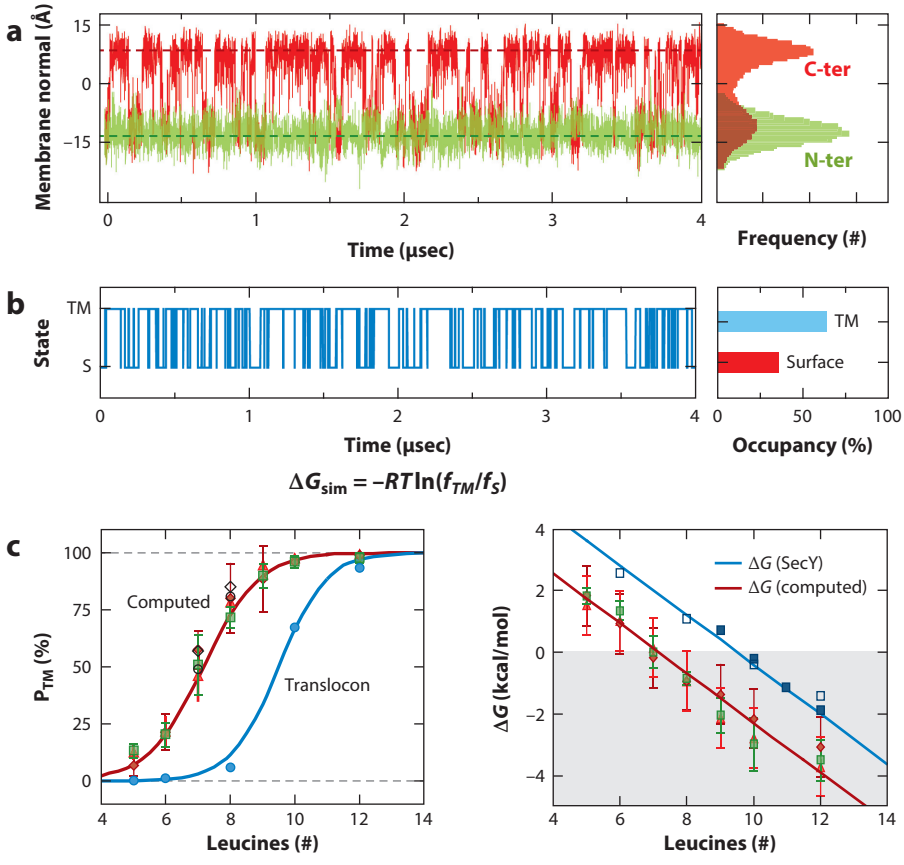


Figure 15

Molecular dynamics (MD) simulations suggest that transmembrane helix insertion involves equilibrium with the membrane interface. (*a*) *Escherichia coli* ribosomes elongate nascent chains at the relatively slow rate of about 20 residues/sec (190). This means that the emerged nascent chain spends about 50 msec exposed to the complex environment of the membrane and translocon before the next amino acid addition. This provides plenty of time for the nascent chain to explore its local environment as it seeks its lowest-free-energy state. For membrane proteins, the local environment must include the membrane interface. The MD simulations of Martin Ulmschneider and Jakob Ulmschneider provided us with important—and provocative—insights into the membrane protein folding problem in the context of the Hessa et al. (42, 43) experiments. The first question was a simple one: What is the trajectory of an unfolded GL_nG peptide when placed in the vicinity of a POPC membrane? The answer is that the peptide rapidly moves to the membrane interface, folds into an α-helix, and divides its time between interfacial and transmembrane states. (*b*) Once equilibrium is reached in a few microseconds, it is easy to calculate the free energy ΔG_{sim} of insertion from the ratio of the average time the peptide spends on the surface and across the membrane. (*c*) Except for a 2 kcal mol^{-1} offset, the simulation results track the Hessa et al. results perfectly. Such simulations have yielded a similar result for peptides of the form $G\text{-}L_nRL_n\text{-}G$ (132). The results strongly imply an important role for the membrane interface in translocon-guided insertion of membrane proteins (130). Figure adapted from Reference 129.

fraction spent spanning the membrane (**Figure 15**). Two findings were highly significant: First, the peptides quickly moved to the membrane surface, never to return to the aqueous phase, and second, the incremental increase of insertion frequency with the number of Leu exactly matched that observed by Hessa et al. (42). The only difference between the simulation and Hessa et al.'s results was a constant offset of one or two kcal mol^{-1}. The point of the first finding—and this is

extremely important—is that greasy peptides have a huge preference for the membrane over the aqueous phase. The melittin results discussed above send the same message. Greasy peptides will always have a high preference for membranes, and the lowest free energy of a greasy peptide in a cell will always be achieved when it associates with the membrane. A cell's problem is the orderly control of such peptides to prevent irreversible aggregation. The translocon makes orderly control possible.

Later, in a second equilibrium simulation, we examined another set of Hessa et al. peptides of the form GL_nRL_nG ($n = 5 \ldots 8$) as a follow-up to our earlier work with the Tobias lab (33, 116). The work confirmed the important role of Arg snorkeling to the interface (65). Importantly, the simulation insertion free energies paralleled the translocon-assisted insertion poly-Leu segments carrying an Arg residue (43). We concluded that the membrane interface plays an important role in translocon-guided insertion (23, 132).

ESCHERICHIA COLI COME TO THE LAB

My collaboration with Gunnar and his lab led me to conclude that future progress required the lab to have a "real" biology component. I talked to Dieter Langosch from Munich Technical University about this at a FASEB research conference in 2005. He recommended one of his graduate students, Eric Lindner, who was just finishing his Ph.D. work on a ToxR-based system for investigating heterotypic TM domain interactions (83, 110). ToxR is a single-span cholera toxin transcription-activator membrane protein that can be used to study dimerization of artificial TM domains engineered into the protein. Eric joined the lab as a postdoc in 2007 and has become our *E. coli* "whisperer." He has a deep understanding of molecular genetics and *E. coli* physiology, as well as a deep sense of adventure, curiosity, and honesty, all of which are essential for working at the frontier.

Our starting idea was to examine translocon-guided TM helix insertion in a different context. In Gram-negative bacteria, the SecA motor protein drives secretion of proteins through the SecY translocon into the periplasm. All periplasmic and outer membrane proteins follow this path. SecA generally recognizes such a protein by virtue of a cleavable N-terminal signal sequence. Gunnar had gained fame by developing algorithms to recognize and define the sequence requirements for SecA recognition of signal sequences (137). Eric and I simply wondered if a hydrophobicity scale obtained via SecA would be the same as one derived using a mammalian cotranslational microsomal system as in the Hessa et al. experiments.

Our first simple-minded idea was to engineer a greasy TM segment into the hydrophobic region of a secreted protein such as maltose-binding protein. We thought that as the greasy segment passed through the translocon on its way to the periplasm, it would partition into the membrane to form a single-span membrane protein. We quickly learned, as Eric says about failed experiments, "*E. coli* don't like that." The rule we learned is that the secreted protein must be natively stable to be secreted by SecA. After trying a lot of other simplistic ideas, Eric began a search for suitable single-span membrane proteins. Thinking about ToxR, he realized that it had a far-downstream TM segment, but no signal sequence, meaning that SecA could recognize signal-sequence-like proteins even if the signal was more than 100 residues downstream from the N terminus.

Eric began the search for other single-span membranes with these ToxR-like characteristics. So far, we've identified six, with the aid of MPex (124), including CadC, which regulates the expression of the cadBA operon, and RodZ, which plays a key role in the maintenance of *E. coli*'s rod shape. We carried out extensive studies of the expression and properties of both proteins (86, 102). The three important general findings are that (*a*) both proteins depend strictly on SecA and are independent of the signal recognition particle (SRP) pathway, (*b*) the introduction of a signal

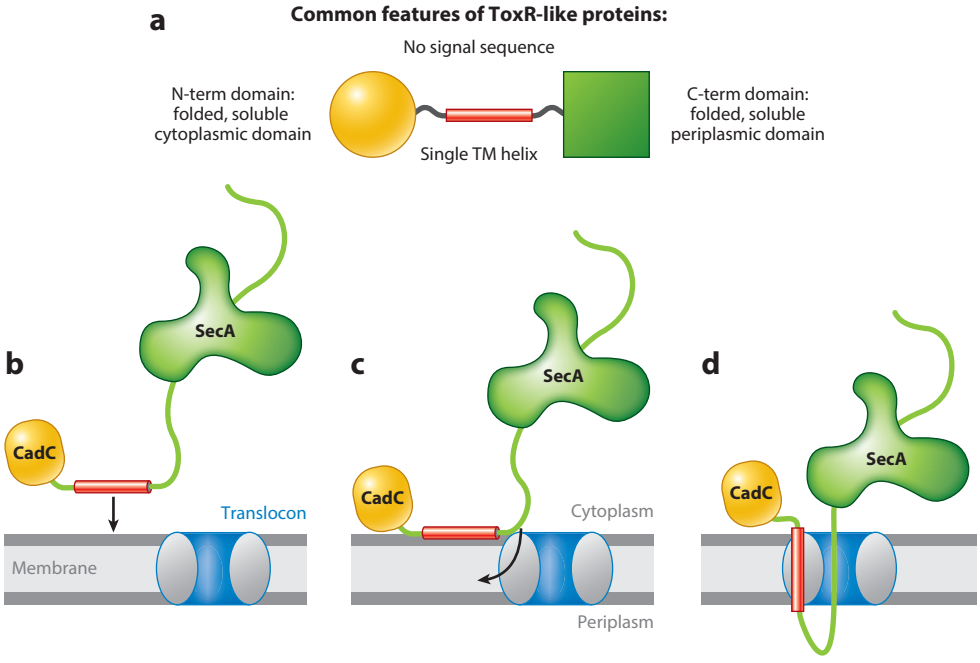


Figure 16

ToxR-like proteins provide a new approach to understanding translocon-assisted transmembrane (TM) helix insertion. Review articles on membrane protein biogenesis and folding inevitably show schematics of polypeptide chains passing smoothly out of the ribosome and into translocons, where they are either passed through the membrane or diverted into it, depending largely upon hydrophobicity. Eric Lindner and I decided to examine this schematic through a different approach. The SecA motor protein of *E. coli* is responsible for moving proteins secreted into the periplasm through the translocon. We wondered if a TM segment inserted into a secreted sequence would partition into the membrane during its translocon passage. The problem with that approach was that the inserted segment disrupts the stability of the secreted protein, leading to its demise by proteolysis. That led Eric to look for proteins like ToxR that have two soluble folded domains connected by a TM helix (shown in panel *a*). He found perhaps a half dozen natural proteins. Importantly, none of the proteins have an N-terminal signal sequence, and all inserted into the membrane only via SecA without the involvement of the signal recognition particle pathway. Furthermore, he found he could create artificial ToxR-like proteins following the prescription summarized in panel *a*. These proteins preclude entirely the usual schematics of translocon-assisted insertion; the soluble, stably folded N-terminal domain appears long before the TM domain. The only way we can see for SecA-driven insertion of the protein to occur is summarized in panels *b*, *c*, and *d* (87). This scheme suggests that binding of the TM segment to the membrane interface is the crucial step in secretion. The role of the translocon is to provide a pathway across the membrane for the polar C terminus of the helix. The process of flipping across the membrane causes "threading" of the translocon, whatever that may mean. Panel *b* adapted from Reference 87.

peptidase cleavage site (-AXA-) at the periplasmic side of the TM helix is useful for studying the stability of TM segments introduced via the SecA pathway, and (*c*) chimeric ToxR-like proteins are easily constructed for various purposes (87) (**Figure 16*a***).

This brings me back to the translocon and a fundamental question: Does the biological hydrophobicity scale measure the free energy of transfer from water to membrane, as I think may be widely assumed? Our various simulations indicated that the water inside the translocon is not bulk water, implying strongly that the biological scale does not measure bilayer-to-water free energies. We have proven this using Eric's experimental approach (87). We constructed Leu_nAla_{16-n}

TM segments and asked how many leucines are required for the TM to partition fully into the membrane from the translocon. The answer is $n = 5$. By introducing an A-X-A cleavage site, we found that Leu_5Ala_{11} inserted via SecA is not stable in the membrane; it drops out of the membrane into the cytoplasm. To keep the segment stable in the membrane after cleavage, n must be 8 or greater. Ergo, $\Delta G_{app} \neq \Delta G_{wbi}$. This means that the fundamental problem of computing membrane protein structure from first principles requires quantitative knowledge of lipid–protein interactions.

THREADING THE TRANSLOCON

The generally accepted description of the insertion of α-helical membrane proteins into the bilayer is that the helices march one after another into the translocon and thence into the lipid bilayer. This scheme clearly cannot describe the assembly of ToxR-like TM helices into the membrane (**Figure 16*b–d***). The helix emerges from the translocon after the soluble N-terminal domain has emerged and folded into a stable protein in the cytoplasm. Logic suggests that in this case the single hydrophobic TM helix likely first partitions onto the surface and then spontaneously adopts a transmembrane configuration (**Figure 15**). The major barrier for the TM movement is the cost of moving the polar C terminus across the membrane. This seems to be the sole purpose of the translocon in this case: It provides a pathway for the polar end of the helix and the following relatively polar chain of the C-terminal domain. Furthermore, this role for the translocon makes sense in terms of the thermodynamic equivalence of the translocon interior and the membrane interface. This thinking leads to a different view (23) of the role of the translocon in the assembly of membrane proteins (**Figure 17**).

HOW DOES SecA WORK?

Given that SecA can insert TM segments independent of the SRP pathway, we need to know just how SecA does the job. There is a huge literature on SecA (22), and great progress has been made structurally (188). But after reading all the papers, we still do not understand crucial details, so we have turned some of our attention to that problem. Of course, as usual, progress depended on the appearance of a new first-rate "out-of-the-blue" postdoc. In this case it was Guillaume Roussel, who received his Ph.D. at the University of Namur (Belgium) in the Theoretical and Structural Physical Chemistry Unit under the direction of Catherin Michaux. Along the way, he had spent time in Mark Sansom's lab at Oxford University learning about MD simulations, as well as doing high-performance computing at the Montreal Heart Institute working on the expression of calcium channels in eukaryotic cells. Another perfect match!

We realized that, to begin to understand SecA, we first needed to understand exactly how it interacts with membranes. Guillaume began by studying the stability of SecA in aqueous solutions that mimic the cytoplasm of *E. coli* (106); then studied the binding of SecA to LUV formed from various lipids (108); and, finally, through a series of clever experiments, found that SecA binds only as a monomer to vesicles formed from *E. coli* lipids, even though it is dimeric in solution (109). Most recently, Guillaume has examined in detail the topology of SecA bound to LUV formed from *E. coli* lipid (107). This is an important step toward understanding how SecA finds its way to the translocon to secrete proteins.

WRITING THE BOOK ON BIOMEMBRANES

Anyone who has studied a particular subject for many decades will eventually be tempted to write a book. In 1994, at the dawn of the revolution in membrane protein structure determination, I

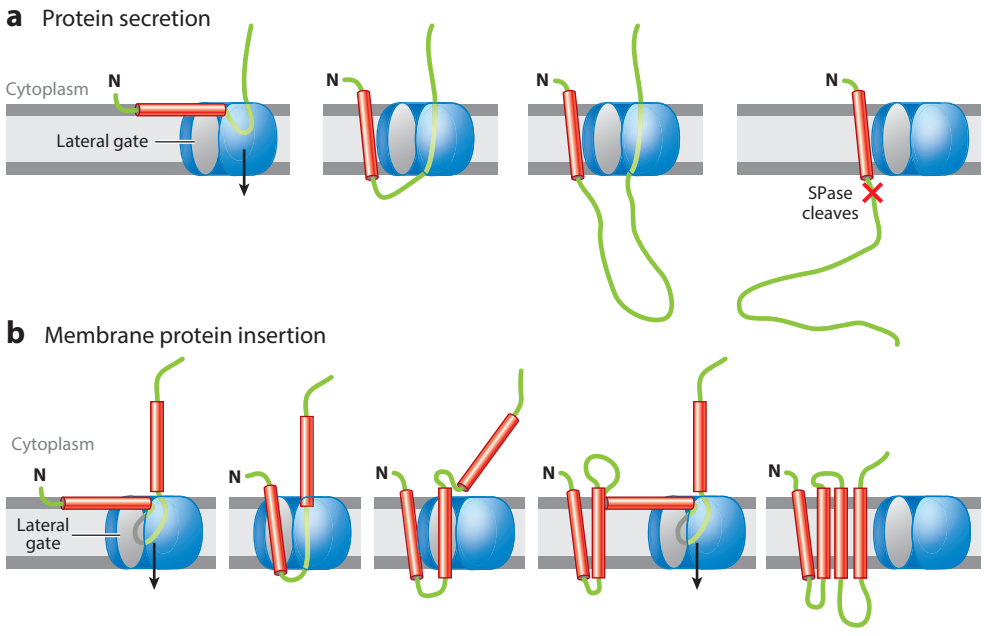


Figure 17

A revised schematic for how translocons work. A new view of how the translocon system might work has emerged over the years from the experiments in my lab and the labs of my collaborators (23). The "translocon pore" is not simple; it is rather tortuous, and the water within is highly restrained. Our cumulative data show that interactions of nascent chains with the membrane interface are likely paramount in the translocon-guided insertion of TM segments. It is especially important to consider the slowness of protein synthesis by the ribosome, and the likely complexity of the energy landscape for folding. Imagine an amino acid segment emerging from an *Escherichia coli* ribosome: The emergent chain has about 50 msec to explore its complex environment formed by the membrane, translocon, and ribosome to find its lowest free energy before the next amino acid is added. Importantly, the energy landscape changes with each amino acid addition. For SecA-assisted secretion of ToxR-like proteins, the membrane interface is crucial (see **Figure 16**). (*a*) A reasonable view is that the translocon simply provides an adaptable a pathway for the more polar segments of the secreted chain without the necessity of passing through the heart of the translocon, as in the schematics. (*b*) This idea readily accommodates the insertion of transmembrane segments. Figure adapted from Reference 23.

edited a volume on experimental approaches to membrane protein structure (154). The experience was eye-opening. For example, I was asked at the final galley-proof stage to edit everyone's introductions to be sure that the latest 3D structures had been referenced. My reward for the effort was a bill for over $1,000 to pay for the resulting redaction charges! Then, when all the chapters were complete, I was sent instructions on how to create the index by identifying key words using 3" × 5" cards. That was too much! There are professionals who do indexing for a living. I suggested that they hire one, which eventually they did. I was subsequently approached about co-authoring with Bob Gennis a second edition of his pioneering 1989 book on membranes (34). That possibility collapsed when I asked for computer files containing text and references (no) and for at least a few color plates (again, no).

These experiences pretty much soured me on writing a book on membranes and their proteins. So, I was not very receptive to the idea of writing one when I was approached by Bob Rogers from Garland Science Press at the 2006 Biophysical Society meeting in Salt Lake City. I told him I would consider it if, among other things, they agreed to full color and minimal scut work. He said,

"No problem." He told me that he had also approached Don Engelman, suggesting co-authorship. Don and I both knew writing a major book would require a huge time commitment, but we agreed that a comprehensive book about cell membranes was needed and figured that it might not be too onerous a task if we shared the load. We put together an outline of planned chapters for review, which Bob sent off to other membrane folk for their opinions of the project. Among them was Gunnar! He was enthusiastic and expressed interest in joining us, which he did. Miracles never cease!

Garland assigned one of their outstanding editors, Summers Scholl, to guide and support us. The four of us gathered at a resort hotel in Vermont in July 2008 to develop a writing plan. Five hundred and forty-six pages and 568 figures later, *Cell Boundaries: How Membranes and Their Proteins Work* (166) was published in January 2022 by CRC Press, which by that time had subsumed Taylor and Francis Group, the parent of Garland Science. Working in partnership with Gunnar and Don over those 14 years has been one of the great pleasures of my career.

ONWARD!

My lab, small as usual, continues to plug away at elucidating fundamental principles of membrane protein folding and biogenesis. Despite 50 years in the research game, I can't give it up. I love the challenge and the adventure of working at the frontiers of membrane biophysics with postdocs and colleagues. My friend Michael Cahalan says there are two kinds of scientists: explorers and map makers. We are explorers. As all scientists know, collaboration is an ongoing imperative in our work. It certainly has been one of the joys of my scientific journey. And I am happy to report that the National Institute of General Medical Sciences has just awarded the lab a five-year research grant to support our next expeditions to the frontier to understand in vivo folding of membrane proteins. It's going to be great fun!

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

I have acknowledged the work of the postdocs that have passed through the lab. I am deeply grateful for their friendship and their enthusiasm for working on difficult and challenging problems. One of the many joys of science is ongoing interactions at scientific meetings with colleagues. I have been blessed over the years with some great colleagues who have shared my enthusiasm for science and influenced my thinking in subtle ways even though I've not written papers with them. You know who you are! I thank all of you for your collegiality and congeniality over the years. I owe a big part of the success of my lab over the years to two lab managers. My first manager was Mike Meyers. Mike was not a formally trained scientist, but he quickly learned what the lab needed. Perhaps his greatest contribution was the language skills he derived from having studied ancient Greek. He edited the lab's papers and taught the postdocs how to write well. Today, the lab is managed by Dr. Gargi Dasgupta, who keeps the lab running smoothly while providing expertise in molecular biology. My wife, Jackie Dooley, a terrific wordsmith, provided many suggestions for improvements in the text of this autobiography. Thanks, Dear! Mr. Paul Neyman has compiled critical events in Columbus, Mississippi (1890–1949) from newspaper articles that provided important background information (94). He graciously provided an Excel database that facilitated searches. I am particularly happy to thank Dr. Jean Chin and Dr. Peter Preusch, now

retired from the National Institute of General Medical Sciences (NIGMS), for their belief in and support of me and the lab over many years. Finally, I am pleased to acknowledge the support of the lab's early research by the National Science Foundation (grants GB-40054, PCM73-00827) and the National Institute of Neurological Diseases and Stroke (grant R01-NS10837) and long-term support by NIGMS via grants R01-GM46823, R01-GM68002, R01-GM74637, P01-GM86685, and R35-GM139652.

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