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A Physical Chemist's Expedition to Explore the World of Membrane Proteins

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NMR of base-stacking interactions in nucleic acids in solution, dynamic structure of bilayer membranes, membrane biophysics, metal cofactor structure and function, redox linkage and proton pumping in cytochrome *c* oxidase, methane hydroxylation by the particulate methane monooxygenase, early kinetic events in protein folding

Abstract

Despite growing up amid humble surroundings, I ended up receiving an excellent education at the University of California at Berkeley and postdoctoral training at Harvard. My academic career at Caltech was shaped by serendipity, inspirational colleagues, and a stimulating research environment, as well as smart, motivated students and postdocs who were willing to join my search for molecular understanding of complex biological systems. From chemical physics I allowed my research to evolve, beginning with the application of NMR to investigate the base stacking of nucleic acid bases in solution, the dynamic structure of membranes, and culminating with the use of various forms of spectroscopy to elucidate the structure and function of membrane proteins and the early kinetic events in protein folding. The journey was a biased random walk driven by my own intellectual curiosity and instincts and by the pace at which I learned biochemistry from my students and postdocs, my colleagues, and the literature and through osmosis during seminars and scientific meetings.

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MY HUMBLE BEGINNINGS

I was born in San Francisco and brought up in relatively humble surroundings. My parents

were both immigrants from Southern China. My father came to the United States as a teenager in the 1920s, and my mother emigrated in her early twenties in 1936. Neither of my parents had any formal education. Both worked in a factory manufacturing denim for Levi Strauss & Co. The working hours were long, as was typical of sweatshops during those days.

Coming from a Chinatown ghetto community, I grew up culturally disadvantaged, if not deprived. I was certainly not destined to be a university professor. There was not a drop of academic blood in my body. Moreover, there were no role models, scholars, scientists, or professionals for me to emulate. I was the first in my family to attend university, receive a college degree, and obtain a PhD. How I ever got to where I ended up makes for an interesting saga. It was mostly serendipity, and not by design or program!

After the Second World War ended in 1945, my parents decided that I should move to Hong Kong to receive a Chinese education. After all, I was growing up to behave like a “hollow bamboo,” without any knowledge or appreciation of my Chinese heritage or culture. So in the spring of 1949, I was thrown into a Chinese middle school in Hong Kong. I had just turned 12, and by age I was assigned to the first year of middle school (the equivalent of seventh grade in the United States). Everything was taught in the native language, including the subject “English.” Not surprisingly, I flunked out at the end of the term. It was a socially humiliating experience, if not a psychological trauma that took many years to shake off.

Ultimately, I ended up in an English-speaking school run by the Irish Jesuits. It was here that my interests in academics were kindled and I developed a fascination for mathematics and science. A number of excellent math and science teachers had aroused my interest in these subjects with their inspiring style of teaching.

In the fall of 1953, I returned to California and entered the University of San Francisco (USF) with the intention of joining the

priesthood and becoming a science teacher. At the age of 16 going on 17, I was not up to the challenge of a rigid lifestyle and I soon gave up the idea of becoming a Jesuit. Instead of continuing my studies at USF, I transferred to U.C. Berkeley with the help of the chairman of the science faculty at USF.

BERKELEY DAYS: FROM UNIT OPERATIONS TO QUARTIC OSCILLATORS

U.C. Berkeley was a difficult school for me. I was not prepared for the rigorous curriculum. Berkeley was also intimidating in other ways. Because I was brought up in a relatively sheltered environment throughout my adolescence, it took some effort to build up the self-confidence to survive in this seemingly “unsupportive” environment. I had to find myself.

Two professors at U.C. Berkeley were inspirational to me, both inside and outside the classroom. Professor George Pimentel’s lucid lectures on chemical equilibrium did much to stimulate my interest in the complex chemistry of solutions. More importantly, he maintained an interest in my overall development as a young man and steered me toward cultivating intellectual outlets other than just chemistry. Professor Andrew Acrivos taught me applied mathematics, chemical engineering thermodynamics, and kinetics, and he did me a greater favor by introducing me to the process of self-study and independent research. Thus, by the time I became a senior, I was prepared to learn things on my own from sources outside the classroom. During my senior year, I began auditing courses in physics and chemistry that were not part of the formal curriculum.

After my graduation from U.C. Berkeley with a BS in chemical engineering in 1957, my original plans were to do graduate studies at the University of Minnesota. For health reasons, I ultimately stayed at Berkeley to work toward a PhD in physical chemistry. Professor Kenneth Pitzer, the then dean of the College of Chemistry, arranged for my admission and late registration. Even so, by the time I was allowed to

register and discuss research with professors in the College, essentially all the vacancies in the research groups I was interested in were filled. I eventually signed up to work in the area of EPR for Professor Rollie Myers, who happened to be on sabbatical leave in Switzerland at the time. Professor William Gwinn was willing to take me on as a graduate student for one year until Dr. Myers returned. A year later, Dr. Gwinn decided to keep me on as his own student, as I was well on my way toward completing my thesis research. Dr. Myers was not aware of this arrangement and outcome until many years later when he was on sabbatical with me at Caltech.

My PhD thesis was on the microwave spectrum of oxetane (or trimethylene oxide) and its molecular structure. The work was concerned with the nature of the potential function describing the out-of-plane vibration of the four-membered ring. At that time the planarity of this heterocycle was controversial. According to the microwave spectrum, the molecule was essentially planar, but the ring was bent according to far-infrared spectroscopy. My thesis work was to reconcile the microwave and far-infrared data. It was possible to fit both sets of data in terms of a potential function consisting of the quartic potential with a small central barrier, with the zero-point vibrational level above the top of the barrier (15). In other words, the four-membered ring of oxetane was essentially planar. The remarkable feature of the fit was how well the far-infrared data were accounted for by the quartic oscillator, including the isotope shifts of the $\Delta n = 1$ transition frequencies when the three CH_2 groups on the periphery of the four-membered ring were replaced by CD_2 . Despite the excellent agreement between theory and experiment, it took almost a decade before the story was finally accepted by the scientific community.

ONWARD TO HARVARD

I was set on an industrial career after obtaining my PhD. In fact, by the early spring of 1960, I had lined up attractive positions at MIT Lincoln Laboratories, Lockheed, General Electric

(to work with Bruno Zimm), and IBM (to work with Enrico Clementi). My PhD mentor, however, had a different plan for me. Upon his insistence, I applied for an NSF Postdoctoral Fellowship to work with Professor Norman Ramsey in the department of physics at Harvard University. High-resolution solution NMR was developing at the time, and Professor Dudley Herschbach, who was a beginning assistant professor of chemistry at Berkeley, suggested that a good way for me to enter the field with my background in microwave spectroscopy of gases was to study the NMR of small molecules in molecular beams. At Harvard, I would learn the physics of NMR rigorously from Professors Ramsey, Edward Purcell, and R.V. Pound. Thus, Ramsey seemed like the perfect choice. Nonetheless, the NSF application was just an exercise for me to keep peace with my professor. When the word came on March 15, 1960, that I had won an NSF Fellowship, I was taken aback. When I accepted to go to Harvard in April, Dr. Gwinn was pleased, but my father was disappointed that I took the \$4500 fellowship instead of the much-higher-paying positions at General Electric and IBM that I was seriously considering. He showed his disappointment by reminding me, "Son, I don't have a PhD, but I make much more money than that."

NMR IN MOLECULAR BEAMS

I arrived in Cambridge, MA, before Labor Day 1960. The Ramsey laboratory had just assembled a molecular beam machine with the universal detector. The mass spectrometer with electron-bombardment ionization to detect the molecular beam allowed molecular beam magnetic resonance studies to be extended to molecules beyond molecular hydrogen. As I was the only chemist in the group, it was incumbent upon me to prepare other molecules for these experiments. Toward this end, I prepared HF, HCl, HBr, HI, and HCN, and the proton spin-rotation coupling constants of these molecules were subsequently determined by Jim Pinkerton, a PhD student in the Ramsey laboratory.

For my own research, I embarked on measurements of the spin-rotation coupling of ^{15}N in molecular $^{15}\text{N}_2$ and the rotational magnetic moment of this molecule (13). Because the ^{15}N nuclear moment and the rotational magnetic moment were one order of magnitude smaller than the corresponding magnetic moments in H_2 , narrower slit widths had to be used in collimating the molecular beam and detecting magnetic resonance, and to make up for the loss in sensitivity of detection of the magnetic resonance signal, the standard single slit was replaced by multiple slits. The ^{15}N spin-rotation coupling constant and the rotational magnetic moment of $^{15}\text{N}_2$ culminated in the determination of the paramagnetic contribution to the ^{15}N nuclear magnetic shielding and the absolute shielding scale for the ^{15}N chemical shift, as well as the paramagnetic contribution to the magnetic susceptibility in molecular N_2 .

It soon became obvious that the molecular beam magnetic resonance method could be extended to molecular systems with larger magnetic moments, e.g., molecules with unpaired electrons. The first system I tried was NO, a diatomic with an unpaired electron in a π -molecular orbital. This electron spin is coupled via spin-orbital coupling to the orbital angular momentum along the N–O bond axis, leading to $^2\pi_{1/2}$ and $^2\pi_{3/2}$ Λ -doublet states separated by 121.1 cm^{-1} . NO in the $^2\pi_{1/2}$ state is non-magnetic, but owing to rapid end-over-end rotation of the molecule, a large rotational magnetic moment is produced (12). Professor J.H. Van Fleck happened to be in the laboratory the afternoon this discovery was made, and by the next morning he had explained the observation using perturbation theory to mix the $^2\pi_{1/2}$ and $^2\pi_{3/2}$ Λ -doublets by electron spin-rotation coupling and the Zeeman interaction of the electron spin. To a novice scientist such as myself, this was an impressive demonstration of real raw intellectual power. The following week I picked up a copy of Van Fleck's book *Theory of Electric and Magnetic Susceptibility* at the Harvard Coop bookstore in Harvard Square and read it from cover to cover. Subsequently, I had many scientific discussions with Dr. van Fleck, and

these encounters left a deep and lasting impression on me, reinforcing that I had made the right decision to do my postdoctoral study at Harvard.

The second free radical I tried to study on the molecular beam apparatus was the methyl radical, which was produced by pyrolysis of tetra-methyl lead in a hot source constructed from a quartz tube. I was able to deflect the $\text{CH}_3\cdot$ in both the dispersing and refocusing inhomogeneous magnetic field regions, but the electron spin resonance elicited in the homogeneous magnetic field region of the molecular beam apparatus was spread out because of the large coupling of the electron spin with the large rotational magnetic fields produced by end-over-end rotation of the radical.

BEGINNING MY ACADEMIC CAREER AT THE UNIVERSITY OF CALIFORNIA AT RIVERSIDE

During the spring of 1961, I was offered the position of assistant professor at the University of California at Riverside (UCR). The graduate program in chemistry at UCR had just started, and I thought I could contribute to building a high-quality program. It was here that my original interest in science teaching was finally beginning to take shape. I also began to develop an interest in the mentoring of graduate students. My graduate training at Berkeley and my postdoctoral experience at Harvard had convinced me that research training was a great way to encourage young people to develop their potential to become original and independent research scientists. Thus, my career as an academic scholar and teacher was beginning to gel. I was about to turn 25.

Although I had finally settled on my career goals, my research plans were far from crystallized. I did not want to continue in the NMR of molecular beams. This type of spectroscopy, like microwave spectroscopy, was maturing and becoming less likely to generate new concepts or breakthroughs. To train graduate students, I felt that the research they worked on had to be “discovery” driven. In curiosity-driven sci-

ence, the young person could be trained to define the scientific question, develop the research plan, formulate the hypotheses to be tested, and design the experiments to test them. So, during my first year at Riverside, I tried a number of areas that were new to me, including the solution structure of transition metal complexes in solution by NMR (introduced to me by Professor Donald Sawyer), NMR of purine and pyrimidine bases (introduced to me by Professor George Helmkamp), and EPR of concentrated alkali metal ammonia solutions. Magnetic resonance in solution appeared to be a developing field, and it was capturing my intellectual attention.

I happened to be at the right place at the right time. It was my good fortune that a Varian DP 60 NMR spectrometer was sitting idle in the Citrus Experimental Station and that it would become the tool of my research. All I had to do was get the spectrometer fired up and running. Before long, I was the faculty member in charge of the spectrometer, the NMR technician, and the NMR operator for the department. I worked together with Richard Kula and Donald Sawyer on the NMR of metal complexes of ethylenediaminetetraacetic acid (EDTA), methyliminodiacetic acid (MIDA), and nitrilotriacetic acid (NTA) (19), and with Marty Schweizer and George Helmkamp on the NMR of purines (87). Oscar Paez started to prepare concentrated alkali metal liquid ammonia solutions, and we determined the microwave skin depth of these metal solutions by EPR to estimate the electrical conductivity (11). Robert Iwamasa started a project on the selective hydration of alkali and alkaline earth metal ions in dilute solutions in acetonitrile.

During this period, I maintained an interest in spin-rotation coupling and the paramagnetic contribution to nuclear magnetic shielding. While at Harvard, I had many conversations with Professor John Baldeschwieler on ^{19}F NMR relaxation. It was apparent that spin-rotation coupling was an important relaxation process for ^{19}F . It turned out that this was the case for ^{13}C and ^{15}N as well.

MOVING ACROSS TOWN TO CALTECH

During the spring of 1961, Professor G.W. Robinson invited me to present a seminar at Caltech. Much to my surprise, I was invited to join the Caltech chemistry faculty the following week. It didn't take me long to decline the offer. As my research program was just about to gel, I did not feel that it was time for me to make a move. I was also reluctant to move to such a high-power place. I wasn't sure that I was up to the challenge. Besides, it could mean a dramatic change in lifestyle for me, more than what I was willing to make. When Professors Harden McConnell and Jack Roberts subsequently asked me to reconsider, and a similar offer was extended to me from the chemistry department at Yale, I decided that perhaps I should consider the long-term impact of a stimulating research environment and high-quality graduate students on the development of my research career. After some soul searching, I finally made the decision to move to Pasadena.

Caltech is an amazing place. As the Caltech logo now says, "There is only one Caltech." Yes, it is a unique place to do science. My colleagues are world class and supportive. The graduate students are first rate. All the way around, there is quality. It is simply a classy institution. I have been associated with Caltech for well over 45 years. Not for a single moment have I ever regretted the decision I made in 1961.

MY FIRST RESEARCH PROGRAM IN BIOPHYSICS: BASE-STACKING OF NUCLEIC ACIDS IN AQUEOUS SOLUTION

Soon after I arrived in Pasadena, I began to consolidate my research program to focus on the application of NMR to address the structure of biological molecules and their interactions in solution. As I was leaving UCR, I was already studying the interactions between nucleic acid bases, nucleosides, and nucleotides in water solution. From studies of the colligative properties of these molecules in aqueous medium, it

was evident that these molecules associate in water. The issue was whether they associate via horizontal hydrogen-bonding or vertical base-stacking via hydrophobic and Van der Waal interactions. I was exploiting the ring current shifts of the nucleic acid bases to distinguish between the two modes of association. Together with Dr. Paul O.P. Ts'o and George Helmkamp, Marty Schweizer and I studied the NMR chemical shifts of the ring protons of purine and 6-methylpurine as a function of concentration (22). Upfield shifts were observed, consistent with base stacking, and the extent of the concentration shifts was consistent with the degree of association implicated by osmotic coefficient measurements (100). This conclusion was received with some skepticism at the time. Dr. Y. Kyogoku, working with Professors Richard Lord and Alex Rich at MIT, had independently shown by infrared spectroscopy that derivatives of A and T, and G and C, formed horizontal complementary base pairs in CHCl_3 , just as predicted by Watson and Crick for double-helical DNA (52, 53). It soon became clear that the difference in solvent, water in the case of the NMR experiments and CHCl_3 in the case of the infrared experiments, accounted for the differences in the solution behavior of these molecules. Thus, nucleic acid bases do stack in aqueous buffer. The same vertical forces must be operative in stabilizing the secondary structures of DNA and RNA molecules to overcome in part the significant entropic penalty required to form complementary base pairs in the double helix.

Following up on this work, James Nelson studied the conformational properties of the dinucleotide ApA in aqueous solution (21); Benedict Bangerter, the dinucleotides ApC, CpA, ApU and UpA (3); and Paulus Kroon, George Kreishman, and James Nelson, the conformational properties of ApApA, ApApApA, and ApApApApA (50). Ben Bangerter and Heinrich Peter also discovered that purine could insert into the ApA stacks and form weak short-lived intercalated complexes with ApC, CpA, and ApU, further demonstrating the importance of base-stacking interactions (14). Ben

was also able to use polyU as a template to form a triple helix from two polyU strands and a vertical stack of adenosine, which was stabilized by Watson-Crick and Hoogsteen base-pairing in addition to the vertical interactions along the adenosine stack (2).

PHASING OUT THE CHEMICAL PHYSICS PROGRAM

Part of the consolidation of my research program included the gradual phase out of my interests in spin-rotation coupling and NMR relaxation. One of the first graduate students to join the Chan laboratory at Caltech was Alan Dubin. He looked for evidence of spin-internal-rotation coupling, the coupling of a nuclear spin to the rotational magnetic field generated by rapid internal rotation of a rotor in molecules containing $-\text{CH}_3$ or $-\text{CF}_3$ tops. We selected benzonitrile for this work. In this molecule, the barrier to internal rotation of the $-\text{CF}_3$ group is sixfold; sixfold barriers are typically very small, a few calories per mole. ^{19}F was chosen because the rotational magnetic fields per unit angular momentum experienced by the three ^{19}F nuclei in the $-\text{CF}_3$ top were expected to be large. Alan, together with Tom Burke, built a pulse NMR machine to measure the ^{19}F NMR longitudinal relaxation rates ($1/T_1$) in benzonitrile and hexafluorobutyne-2 (10, 33). The ^{19}F T_1 s were short; moreover, part of the NMR relaxation was observed to be independent of the viscosity of the medium, as expected for spin-internal-rotation coupling. Fluctuations in the rotational magnetic field due to internal rotation should be inertially controlled. As predicted, Alan quenched the spin-internal-rotation coupling in the case of benzonitrile by introducing a substituent at one of the *ortho* positions of the aromatic ring, converting the barrier from sixfold to threefold (33). Threefold barriers to internal rotation are typically 1000 times higher. Additional confirmation of this mechanism of NMR relaxation came from a study of the pressure dependence of the NMR relaxation rates by Professor Jiri Jonas and his coworkers at the

University of Illinois, Urbana-Champaign (9). Later, Charles Schmidt established the importance of ^{13}C spin-internal-rotation coupling in the ^{13}C NMR relaxation of the methyl top in toluene (84).

As I began to embark on the new field of biophysics, it was not obvious to me that my background in chemical physics would serve me well in my future endeavors. Moreover, given that I have had no formal training in biochemistry or biology, I was entering the field with a major handicap. Molecular biophysics was a relatively virgin field, and not knowing much about it was a blessing in disguise. I learned the biochemistry as my interests and understanding developed, and I had no choice but to focus. The more serious hurdle was finding out what was known and what was not known, as well as identifying the scientific questions that needed to be solved and the tools that would get me to the heart of the matter. Here, my physical background and experience in molecular spectroscopy, particularly NMR and EPR, proved to be invaluable. My training in physics armed me with the insights to formulate and analyze complex problems rigorously from the outset. I was also in a better position to develop reasonable conceptual models and hypotheses to test in the laboratory.

FORAYS INTO MEMBRANE BIOPHYSICS

Dynamic Structure of Lipid Bilayer Membranes

In 1968, Michael Sheetz joined my laboratory. He was interested in developing biophysical methods to probe the structure and dynamics of biological membranes consisting of both phospholipids and membrane proteins. The impetus of the work actually came from Professor S.J. Singer of the University of California at San Diego, who was eager to seek more direct physical evidence for the fluid mosaic model of biological membranes (93). Mike initiated a collaborative effort: The Singer laboratory prepared red blood cell ghosts and Mike examined

T₁: longitudinal spin relaxation time

T_2 : transverse spin relaxation time

the proton NMR of the ghost membranes. A Varian HR 220 superconducting NMR spectrometer had just been installed at Caltech, and it seemed like the timely experiment to try.

No discernable features were apparent in the NMR spectrum under ambient temperatures. However, as the temperature was raised to 40°C and above, reproducible resonances appeared characteristic of denatured proteins (41). These resonances originated from spectrin, the major cytoskeletal protein in the membrane of the red cell ghosts (92). Surprisingly, no phospholipid features were evident, even though the lipid bilayer was supposed to be fluid. The obvious conclusion was that the local motions of the lipid molecules were restricted and slow in the membranes; the overall rotation of the ghost was too slow to average out any residue magnetic dipolar interactions among the proton spins.

To address this issue, Mike prepared single-walled phospholipid bilayer vesicles, fractionated the vesicles according to size, and recorded their proton NMR spectra. The proton NMR of the smallest bilayer vesicles, which were 250–300 nm in diameter, was extremely sharp, resembling the spectrum of a small hydrocarbon droplet in a micelle of the same diameter. Moreover, the line widths were not influenced by the viscosity of the medium in which the vesicles were suspended. Thus, the overall tumbling of the vesicle was not a controlling factor in limiting the line widths of the lipid resonances in the small sonicated vesicle. We surmised that the observed fluidity arose from disorder in the packing of the phospholipid molecules to fit the extreme curvature imposed by the bilayer structure (91). Although this explanation of the unusual fluidity of small lipid vesicles seemed intuitively obvious, the curvature effect remained controversial for many years.

An outcome of the curvature effect was that the two monolayer leaflets of a small bilayer vesicle were intrinsically nonequivalent owing to differences in the magnitude and sign of the curvature experienced by the inside- and outside-facing lipid molecules. This asymmetry was subsequently demonstrated by Joseph

Schuh, Utpal Banerjee, and Luciano Mueller (85) and Kenneth Eigenberg (34) in proton NMR experiments at 500 MHz that revealed different chemical shifts for the choline methyl groups of the lecithin molecules occupying the two halves of the bilayer vesicle.

During the 1970s, the Chan laboratory devoted considerable effort toward comparing the properties of single-walled bilayer vesicles with those of multilamellar dispersions. As opposed to single-walled bilayer vesicles, the multilamellar structures corresponded to the global thermodynamic free-energy minimum of the phospholipid-water dispersion. In contrast, single-walled bilayer vesicles were metastable and they tended to fuse with one another to form larger structures. This vesicle fusion was catalyzed by local defects in the bilayer structure. Rüdiger Lawaczeck, Jean-Luc Girardet, and Masatsune Kainosho introduced structural defects into the bilayer membranes by sonicating phospholipid dispersion below the phase transition temperature of the lecithin dispersions (56). These structural defects also mediated transmembrane solute and ion transport (57), reminiscent of the structural fluctuations found in lipid structures containing coexisting gel and fluid domains. Arthur Lau also discovered that amphipathic peptides like alamethicin could mediate the rapid fusion of small bilayer vesicles prepared by sonication above the thermal transition (55). ^1H NMR experiments by Arthur (54) and ^{31}P and ^2H -NMR experiments by Utpal Banerjee, Robert Birge, and Raphael Zidovetzki (1) showed that these peptides were surface active and could serve as nucleating centers to lower the activation energy for vesicle fusion.

To compare the dynamic structure of the bilayer vesicle with that of the multilamellar phospholipid dispersion, the Chan laboratory appealed to NMR line width and relaxation measurements, exploiting the different sensitivity of the T_1 and T_2 to molecular motions of different timescales. Unlike the high-resolution liquid-like spectrum observed for the small lipid vesicle, in lecithin multilamellar dispersions, the choline methyl resonance, the resonances of

the methylene protons, and the terminal methyl protons of the acyl chains were significantly broader in the ^1H NMR spectrum. In addition, the spectrum exhibited intensity anomalies expected for a powder spectrum of a system with motional restriction (23). To estimate the degree of motional restriction, Gerry Feigenson and Charlie Seiter recorded the early part of the free induction decay (FID) in the pulsed ^1H NMR of the lecithin dispersion (17).

Charlie Seiter interpreted the decay in terms of the magnetic dipolar interactions among the methylene protons near the top of the acyl chains that were not averaged by molecular motions, including contributions from both the geminal dipolar coupling as well as intergeminal interactions. To account for the observed motional averaging of the ^1H - ^1H dipolar interactions, he suggested rapid lipid or chain motion about the normal to the plane of the bilayer membrane together with off-axis motion within a restricted range of $\sim 40^\circ$ within the timescale of the NMR measurement (89). Although this analysis was relatively crude, it was an important beginning because it introduced the boundary conditions under which the NMR of membranes needed to be interpreted.

In due course, the ^1H NMR experiment was superseded by ^2H NMR, in which the orientational order of the acyl chains was determined by the extent of motional averaging of the ^2H electric quadrupolar interactions of the C-D bonds within each of the C-D₂ segments (88). The ^2H NMR method became the method of choice because it allowed direct determination of the order parameters of the various methylene segments along the acyl chains, providing a much more precise picture of the flexibility gradient along the hydrocarbon chains.

Nils Petersen elaborated on the motional model of Seiter by introducing the possibility of *trans-gauche* isomerization of the methylene segments along the acyl chains, in addition to the restricted fluctuations of the lipid chains about the director of the membrane (79). In principle, many types of motions could contribute to the reduction of the orientational order of the individual segments of the acyl chains

in a partially disordered lipid system, including *trans-gauche* isomerization, the formation of kinks via β -coupled *gauche*⁺-*trans-gauche*⁻ isomerizations, diffusion of the kinks up and down the acyl chains, dynamic tilting of the hydrocarbon chains, and the entire phospholipid molecule about the membrane director, as well as elastic waves arising from undulations of a collection of phospholipid molecules within the thin bilayer film (74). The formation of ($\pm$) *gauche* bonds should be facile and highly probable, particularly toward the tails of the hydrocarbon chains. The chain fluctuations were expected to be slower, but these motions could involve a single lipid molecule undergoing restricted motion within its accessible volume, as well as a cooperative domain of many molecules undergoing highly damped surface undulations within the lipid film. The timescales of these motions extended from relatively rapid individual molecular fluctuations to slow collective motions of larger amplitudes. Any disorder in the molecular packing of the phospholipid molecules would reduce the cooperativity and increase the amplitude of the restricted motion and the frequency of the fluctuations. In contrast, cholesterol introduced considerable molecular ordering into the motional state of lecithin bilayer vesicles (49).

In those days it was popular to attribute the reduction of the orientational order of the hydrocarbon chains solely to *trans-gauche* isomerization. ^{13}C T₁s and ^2H NMR order parameters and T₁s were interpreted in this manner (58, 98). Gerry Feigenson measured ^1H T₁s of lecithin multilamellar dispersions over a range of temperatures and NMR frequencies and concluded that there were at least two types of motions involved: one correlation time of the order of 10^{-9} s and the other of the order of $\geq 10^{-7}$ s (40). He attributed the faster motion to kink diffusion and the slower motion to chain fluctuation. Masatsune Kainosho, Paulus Kroon, Rudiger Lawazcek, and Nils Petersen (47) and the group headed by Mel Klein at U.C. Berkeley also measured the T₁s and T₂s of the protons of the headgroups and the hydrocarbon chains in small sonicated lecithin bilayer

vesicles (44). From the temperature dependence and the NMR frequency dependence of T_{1s} , it was concluded that the formation and diffusion of kinks occurred on the timescale of 10^{-10} to 10^{-9} s, but the chain fluctuations occurred in the vicinity of 10^{-8} s (74). As expected, the correlation length of a cooperative domain was substantially shorter in the disordered bilayer vesicle.

In these systems, the observed intrinsic T_2s were always substantially shorter (typically by a factor of 10) than the observed T_{1s} , reflecting the more complex power spectrum of the fluctuating magnetic dipolar fields brought about by the complex motions highlighted above. Thus, the dynamic structure of a bilayer membrane is highly complex and the motions are characterized by a hierarchy of motions of different amplitudes and timescales. The Chan group exploited the sensitivity of multiple-frequency NMR measurements to different timescales to sample these motions during the 1970s (8).

Lipid-Protein Interactions and Lipid-Mediated Protein-Protein Forces

According to the fluid mosaic model of Singer & Nicolson (93), the structure of a biological membrane consisted of a fluid lipid bilayer with membrane proteins embedded and anchored vectorially in the thin film. For the embedded proteins to be thermodynamically stable, the protein must be folded with the hydrophobic residues exposed to the lipid molecules, and there must be matching of the hydrophobic hydrocarbon chains to the hydrophobic surface of the proteins (75, 77, 78). Any hydrophobic mismatch could be alleviated by adjusting the number of *gauche* bonds toward the tails of the hydrocarbon chains of the phospholipids, tilting the phospholipids and the hydrocarbon chains about the normal to the bilayer plane, and redistributing the otherwise heterogeneous distribution of lipids in the cell membrane to bring lipids with the appropriate hydrocarbon chain lengths to form the boundary lipids

at the interface of the proteins (78). Thus, any hydrophobic mismatch would necessarily perturb the lipid bilayer and lead to protein-protein forces. Owicki, McConnell, and colleagues were the first to treat this problem theoretically (72, 73). Tim Pearson and Jay Edelman extended this work and showed that these lipid-protein interactions did not readily damp out because of the two-dimensional structure of the bilayer film (77). Correlation lengths were long, of the order of 10 nm. Accordingly, at the appropriate protein concentrations in the membrane, when the perturbed lipid domains overlapped, there could be lipid-mediated protein-protein forces and the protein pair radial distribution function would be modified from the statistically random distribution.

Tim Pearson was the first to look for experimental evidence in support of this prediction. Working with Barbara Lewis and Don Engelman, he examined the protein distribution in recombinants of bacteriorhodopsin in bilayer dispersions formed from lecithins with different acyl chain lengths by freeze-fracture electron microscopy (78). Indeed, the pair protein distribution function differed from the statistically random distribution and varied in a manner predictable by the hydrophobic mismatch at the lipid-protein interface. Both attractive and repulsion interactions were implicated depending on the nature of the mismatch. Subsequently, Tim Pearson extended the study to protein-lipid recombinants of cytochrome *c* oxidase (CcO) and reported evidence for a similar lipid-mediated protein-protein force (76).

Later, Paula Watnick, Phoebe Dea, and Luciano Mueller studied the effects of the transmembrane peptide gramicidin A on the dynamic properties of the bilayer membrane (65, 104). Different deuterated lecithins of varying chain lengths were examined by 2H NMR. As expected, gramicidin A had no noticeable effects on the 2H T_{1s} . However, there were dramatic effects on the homogeneous T_{2s} of the 2H (65). These results were consistent with the expected effects of the incorporated peptide on the elastic constants and the lateral cooperativity of the bilayer structure (104). The peptide

reduced the overall effective correlation length of the cooperative domain.

THE ERA OF MEMBRANE PROTEINS

Cytochrome *c* Oxidase: A Redox-Linked Proton Pump

Our early attempts to study lipid-protein interactions and to obtain evidence for the boundary lipid were carried out using CcO from bovine heart. This work was carried out in collaboration with Professor Tsao E. King and Drs. Chang-An Yu and Linda Yu. Chang-An and Linda Yu (110) provided the laboratory with high-quality protein, and my postdoc Dr. Ming-Chu Hsu and graduate student Valerie Hu prepared lipid combinants of the CcO for NMR studies. Unfortunately, we were unsuccessful in obtaining reproducible-quality ^1H NMR data. Moreover, the NMR signals were broad, presumably because of the difficulty in obtaining a sufficiently homogeneous system and because of chemical exchange effects arising from sampling of the phospholipids over domains of different sizes and compositions. After about one year, we decided to move on to study the protein itself.

At the time, CcO was not molecularly characterized. The subunit composition was still unclear. The protein was thought to contain two iron heme As and two copper ions (4). The hemes were called cytochrome *a* and cytochrome *a*₃, and the copper ions were called Cu_A and Cu_B. It was established that the enzyme participated in dioxygen reduction and was thus the terminal enzyme in the respiratory chain of mitochondria. But little was known about the electron transport and the dioxygen chemistry mediated by the enzyme, not to mention the role played by the metal cofactors in mediating this chemistry. Märten Wikström had obtained the first evidence that CcO might be a proton pump (106), but the issue was highly debated. In any case, the general consensus then was that CcO was a highly complex molecular machine.

A molecule of this complexity was certainly beyond the training and expertise of a physical chemist. In fact, an eminent membrane biologist thought that there was no room for a physical chemist in this field and he advised me to apply my knowledge of physical methods to work on better-defined systems. On the other hand, several laboratories, including those of Helmut Beinert and Tsao King, among others (43, 110), were beginning to isolate the enzyme from mitochondria and purify the protein to homogeneity. The methods of molecular biology were also becoming available at the time, and my instincts suggested that it was only a matter of time before this membrane protein was going to be cloned and overproduced. With the field at this stage of development, it was a perfect opportunity to train graduate students and postdocs. They could learn to define the issues that needed to be addressed, come up with the methods of the solution, and contribute to molecularizing the problem, which was the goal of my jumping into the quagmire to begin with.

The first attempts by the Chan laboratory were directed toward characterizing the metal centers of CcO. National synchrotron radiation facilities had just been established, and X-ray absorption spectroscopy seemed like a good way to identify and characterize the Fe and Cu atoms in the enzyme. Dr. Bob Shulman of Bell Laboratories contributed part of his beam time at the Stanford Synchrotron Radiation Laboratory to allow Valerie Hu and me to perform these experiments. Strictly speaking, we had to obtain Dr. Bill Blumberg's blessings before undertaking the Cu K α -edge experiments, since the turf was divided up into Fe and Cu X-ray absorption between the Shulman and Blumberg groups at Bell Laboratories, respectively. Fortunately, I was able to forestall a political crisis because Dr. George Brown of Bell Laboratories was helping Valerie and me with the experiments.

The interpretation of the Fe K α -edge of CcO was straightforward. However, the Cu K α -edge suggested that one of the two copper ions was Cu¹⁺ when the enzyme was fully oxidized, or the ligand environment of this copper

ion was highly covalent (45). This observation suggested that cysteine sulfurs were associated with this copper cofactor. During those times, the enzyme was thought to contain two iron and two copper ions, and it wasn't until 20 years later that Caughey and coworkers (109) discov-

ered that the correct metal content was two iron and three copper ions. It is now known that Cu_A is really a mixed valent Cu(I)Cu(II) species bridged by two thiolates in the oxidized enzyme (**Figure 1a**) (46, 101, 102). Thus, the Cu $K\alpha$ -edge was giving us the right story, though we

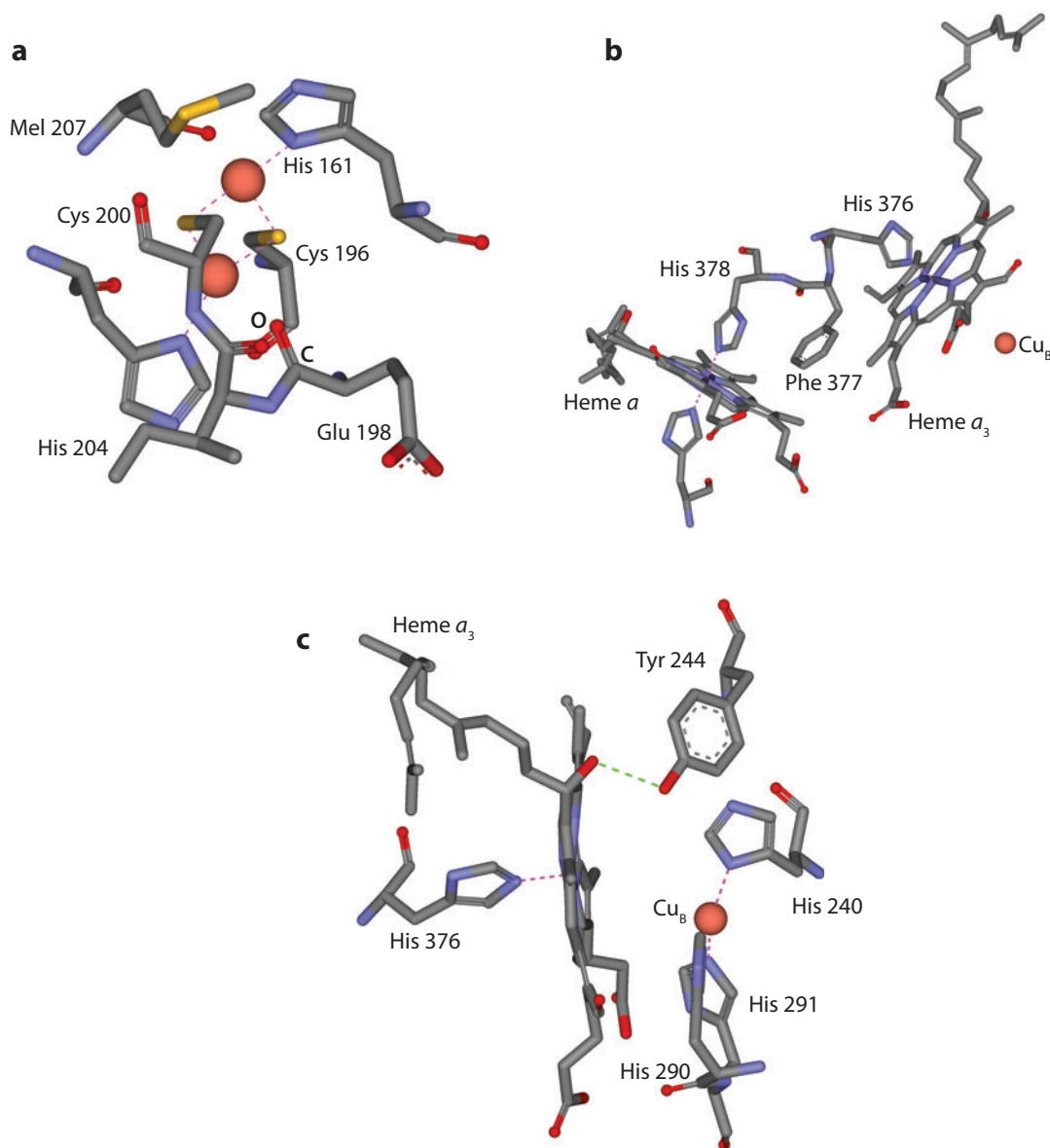


Figure 1

Ligand structures of (a) Cu_A , (b) cytochrome *a* and cytochrome *a*₃, and (c) cytochrome *a*₃ and Cu_B in bovine cytochrome *c* oxidase. Taken from Reference 101.

could not distinguish between a reduced copper and a highly covalent Cu(II) during those earlier times. It turned out the answer was both.

In the meantime, Gary Brudvig, Tom Stevens, and David Bocian joined the CcO project. They decided to use NO to probe the dioxygen-binding site of the enzyme. At the time, the dioxygen reduction site was known to consist of cytochrome a_3 and Cu_B , but this site was EPR silent in both the fully oxidized and reduced forms of the enzyme. In the fully reduced form, both cytochrome a_3 Fe^{2+} and Cu_B^{1+} were diamagnetic, but in the fully oxidized state, the high-spin cytochrome a_3 Fe^{3+} and Cu_B^{2+} were thought to be exchange coupled to yield an $S = 2$ electronic state, which did not possess a Kramer's doublet to facilitate EPR observations. On the other hand, NO was often used as a probe of dioxygen-binding sites. With its unpaired electron spin, the NO could be used to convert an integral spin system into an electronic state with a Kramer's doublet. Armed with this strategy, Gary, Tom, and David treated the reduced and the oxidized oxidases with NO to explore the structure of the dioxygen reduction site (95). EPR signals were elicited for all the cofactors in one or the other of these experiments. In a neat experiment, in which they treated the azide adduct of the oxidized enzyme with NO, they demonstrated that the reaction of NO with the azide adduct led to disproportionation of the azide adduct to give N_2O and N_2 , followed by reduction of the cytochrome a_3 and binding of a second NO to give an exchange-coupled cytochrome a_3^{2+} -NO, Cu_B^{2+} triplet species. The resulting zero-field splitting suggested a NO- Cu_B^{2+} interspin distance of 3.5 Å or a Fe Cu distance of ~ 5 Å for the dioxygen reduction site. This result indicated that the dioxygen-binding site on cytochrome a_3 was located on the same side of the porphyrin plane as Cu_B , providing the first direct evidence that the binuclear site could be bridged by a peroxide intermediate during the initial stages of dioxygen activation (**Figure 1b,c**).

Tom Stevens and Craig Martin moved the project forward another notch by obtaining in-

formation on the ligands of the various metal cofactors. The prevailing view at the time was that this information was needed before the redox potentials and electron transfer rates mediated by the metal cofactors of the enzyme could be understood. With the assumption that these ligands must be highly conserved, Tom and Craig exploited amino acid auxotrophs to incorporate selected amino acids with isotopic labels into the CcO to reveal the identity of the ligands by the nuclear superhyperfine interactions in the EPR that could be elicited from each of the four metal cofactors, either by conventional EPR experiments or electron nuclear double resonance (ENDOR) measurements of the various EPR signals. Thus, ^{15}N ring-labeled histidines and ^2H - and ^{13}C -labeled cysteines were used to obtain yeast CcO when appropriate auxotrophs of *Saccharomyces cerevisiae* were cultured. In this manner, Tom and Craig systematically identified the histidines of cytochrome a , cytochrome a_3 , Cu_A and Cu_B , and the cysteines of Cu_A (6, 63, 96, 97) (**Figure 1**). That cysteines were associated with Cu_A was particularly significant, and judging from the extended X-ray absorption fine structure (EXAFS) in X-ray absorption experiments, thiolates were implicated as the bridging ligand at the binuclear site by other investigators. Because there were only two conserved thiolates in the subunits containing the Fe and Cu cofactors (subunits I and II), and these cysteines were in subunit II, the Chan group had insisted that Cu_A was located in subunit II and that the two cysteines were associated with this site. This controversy over the cysteines persisted for many years until the crystal structure of CcO was finally obtained. The two thiolates provided the bridging sulfurs for the mixed valent Cu(I)Cu(II) Cu_A structure (101).

The Chan laboratory has also contributed to the understanding of the mechanism of dioxygen activation of CcO, which is paramount to the enzyme's function as a proton pump. The binding of dioxygen to CcO had been well studied by many groups ever since the enzyme was identified and purified. Britton Chance championed the method of flash photolysis at low

temperature to study the formation of the dioxygen adduct and the subsequent formation of the peroxide intermediate (Compound C) (27). Malmström and coworkers (32) studied the transfer of the “third” electron to the Compound C by incrementally increasing the temperature, monitoring the electron transfer process by low-temperature UV-visible spectroscopy. David Blair and Steve Witt (7, 25) followed the reduction of Compound C by rapid quench 4 K EPR. Surprisingly, two dioxygen intermediates at the three-electron level of reduction were identified in the low-temperature experiments, with the first intermediate undergoing a bond-breaking step to yield the oxyferryl species. These findings confirmed that the O–O bond in dioxygen was cleaved at the three-electron level of reduction, as in the case of cytochrome P₄₅₀, rather than after the succession of two two-electron reductions as proposed by Malmström and coworkers (32). The formation of the peroxide intermediate and the oxyferryl during dioxygen reduction provides for the two high-potential intermediates that are needed to accomplish the redox linkages during the proton pumping mediated by the enzyme (20, 105).

During the 1980s, the evidence for CcO as a proton pump became increasingly compelling (105, 106). Certain properties of the proton pump were also becoming evident. From thermodynamic reasoning, the transfer of the first two electrons from cytochrome *c* to the dioxygen reduction site via cytochrome *a* and Cu_A could not be directly linked to the pumping of protons, unless the protein had been energized in an earlier redox linkage step and the transfer of these electrons to the oxidized enzyme was to dissipate any residue conformational excitation of the protein to eject the protons that have already been moved across the osmotic barrier. There is simply insufficient driving force to move a proton uphill across the osmotic barrier otherwise (20). Redox-linked proton pumping in CcO is a highly complex process consisting of electron input, electron transfer, proton uptake, redox linkage, and proton ejection steps (86). However, without redox linkage there can be no transfer of redox energy from

the electron degrees of freedom to the protein degrees of freedom, and therefore no proton pumping (66, 67). The putative redox linkage could occur only during the second half of the turnover cycle when the final two electrons are passed to the activated dioxygen, i.e., when the peroxide and the oxyferryl intermediates are presumably formed (67). The redox potentials of these dioxygen intermediates are of the order of 1 volt, which is approximately 700–800 mV higher than the intrinsic redox potentials of the cytochrome *a*₃ and Cu_B without the dioxygen bound (105).

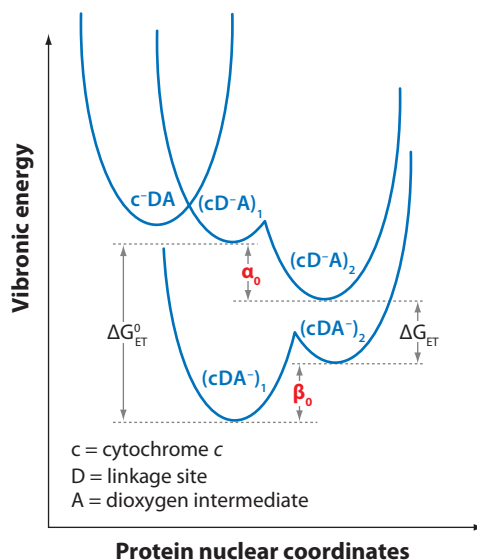
To achieve this redox linkage, the flow of both electrons and protons must be gated. Electron transfer without linkage corresponded to an electron leak without transfer of redox energy to the protein. Without proton gating, protons cannot be effectively pumped because of proton backflow (molecular slip). David Blair and Jeff Gelles (5) introduced the concept of electron gating and emphasized its importance for the robust functioning of a redox-linked proton pump. Siegfried Musser (66) emphasized the importance of proton gating. He also developed the ideas of redox linkage in some detail by embodying the rules in several examples with varying driving forces for proton pumping (**Figure 2**). Molecular models were also highlighted to illustrate these principles (67). At this juncture, the rules remain untested. Although the structure of CcO has been available for almost two decades (46, 102), the experimental testing of these ideas has been slow. We require a detailed understanding of the structural changes in the enzyme when the dioxygen-binding site has become activated and when one of the low-potential centers has been reduced.

In principle the electron transfers linked to proton pumping could originate from either cytochrome *a* or Cu_A. In recent times, the electron flow within the enzyme has been thought to follow the canonical pathway for every step regardless of the state of activation of the dioxygen reduction site: cytochrome *c* → Cu_A → cytochrome *a* → dioxygen-binding site. This need not be the case. In fact, no hard data bear on the transfers of the final two electrons,

the two electrons linked to redox linkage. The Chan laboratory has suggested that the electron flow is bifurcated, with the first two electrons following the canonical pathway, and the second two electrons involved in redox linkage occurring through a different pathway bypassing cytochrome *a* (**Figure 2**).

As noted above, the direct transfer of the latter two electrons would occur with high driving force, and in fact, if the driving force is sufficiently high, the rate could be governed by the Marcus theory of electron transfer on the right side of the inverted region in the plot of k_{et} versus driving force (62). Thus, the Marcus theory could be exploited to gate the electron flow. If the reduction of Cu_A is linked to a conformational change within the protein that significantly raises the reduction potential of the site (**Figure 2**), then the putative electron transfer from Cu_A to the dioxygen intermediates anchored at the binuclear center could be rendered more facile following the redox linkage. In this scenario, the reduction potential of Cu_A would become higher than that of cytochrome *a*, forestalling the electron transfer from Cu_A to cytochrome *a*. In the absence of redox linkage, the electron equilibration between Cu_A and cytochrome *a* would be rapid, of the order of 20,000 s (64), and possibly even faster (113). Aside from the redox potential difference between these two redox centers, the kinetics of the electron transfer between Cu_A and cytochrome *a* could also be tuned by subtle conformational changes in the enzyme.

It goes without saying that there must also be allosteric coupling between the Cu_A and the activated binuclear site in this model for the enzyme to distinguish between proton-pumping and nonproton-pumping events. The Cu_A site, which consists of a Cu(I)Cu(II) bridged by two thiolates, is electron rich and is poised to act as a conformational trigger upon reduction by an additional electron (67). We expect conformational changes at the dioxygen reduction site as well in order to optimize the biological energy transduction (**Figure 2**). The idea is that following redox linkage, the electron transfer from Cu_A to the dioxygen intermediate(s) will



Coupled reaction path (proton pumping occurs):



Uncoupled reaction path (proton pumping does not occur):



Figure 2

Schematics of redox linkage occurring during proton pumping by cytochrome *c* oxidase (CcO). In this model, both Cu_A and the dioxygen-activated cytochrome a_3 and Cu_B are involved in the redox linkage. The primary electron donor that initiates the process is cytochrome *c*, which is denoted by *c*. *D* denotes the electron donor for the electron transfer coupled to the proton pump (i.e., the input linkage site), and *A* represents the peroxide or oxyferryl dioxygen intermediate (electron acceptor) (i.e., the output linkage site). Proton-pumping and nonproton-pumping conformational states of the CcO complex are highlighted by the subscripts 2 and 1, respectively. These symbols are used in unison [e.g., $(c^-\text{DA})_1$, $(c\text{D}^-\text{A})_1$, $(c\text{D}^-\text{A})_2$, $(c\text{DA}^-)_2$] to denote a specific redox and conformational state of the CcO complex. Plotted along the ordinate is the total vibronic energy of the enzyme complex, the electron donating cytochrome *c*, and the protons involved in the dioxygen chemistry and the proton-pumping process. The abscissa denotes the protein nuclear coordinates. α_0 and β_0 denote the amounts of redox energy transferred to the protein to excite the vibrational degrees of freedom during the redox linkage from the input and output redox linkage sites, respectively. ΔG_{ET} denotes the driving force for electron transfer. Figure adapted from Reference 66.

occur with a driving force on the left side of the inverted region to allow the process to be completed efficiently and irreversibly.

CcO can pump up to 1 H^+/e^- (105), and this H^+/e^- ratio decreases with increasing

protonmotive force. The enzyme seems to behave as if there is a built-in clutch to switch the proton-pumping machinery between productive and nonproductive cycles (**Figure 2**). In this manner, variable H^+/e^- ratios could be obtained depending on the external conditions under which the pump must operate.

Membrane Proteins that Mediate Ion and Solute Transport

Aside from CcO, the Chan group has maintained a strong interest in other membrane transporters that mediate ion transport as well as the transport of other solutes. Our interest in ion transporters actually started with the structural studies on nonactin, valinomycin, and alamethicin. This work was inspired by Professor Max Delbrück, whose interests in the biophysics of membranes did much to inspire me to initiate research on the structure and dynamics of the bilayer membrane and the mechanism of ion transport across membranes.

During the late 1960s Jim Prestegard worked on the structure of nonactin and tried to determine the molecular basis of the ion selectivity for K^+ . Jim carried out ion-binding studies and concluded that the difference in the free energy of hydration and complexation of the naked cation by the nonactin provided the driving force for the selectivity (80, 81). The same result was obtained in the case of valinomycin. However, it was generally assumed that the selectivity was determined by the ability of the polar $C=O$ and ether oxygen groups on the transporter to adapt and accommodate the naked cation. The size of the cation contributed to the selectivity to the extent that it determined the ability of the macrocyclic ligand to adapt and bind to the cation to attain the optimal free energy of complexation. Ion selectivity was actually determined by the difference in this free energy of complexation of the naked cation and the free energy of hydration of the same cation in the bulk solvent.

When Joseph Falke joined the Chan laboratory, he wanted to work on the anion trans-

porter in the red cell membrane. He asked me to come up with a biophysical method to probe the Cl^-/HCO_3^- exchange, and I suggested ^{35}Cl and ^{37}Cl NMR. Joe Falke exploited this technique to study the binding of Cl^- to band 3 (38). He prepared ghost membranes with both the inside-facing and outside-facing sides of the ghosts exposed to a Cl^- solution, and ghost membranes with either the inside- or the outside-facing side exposed. Joe was able to recruit all the sites to one side only by using a reversible transport inhibitor (37, 39). By using the arginine-specific reagent glyoxal to modify the transporter covalently, he argued that the transport site was an arginine, which was alternatively exposed to the inside-facing and outside-facing sides during the transport cycle. Thus, the anion transport occurs via an alternating transport mechanism, or the ping-pong mechanism (36). Following up on Joe Falke's work, Kathy Kane tried to identify this arginine transport site on the primary sequence via ^{14}C radioactive labeling. The band 3 was cleaved by specific proteases after chemical modification, and attempts were made to separate the transmembrane peptides by reverse phase hydrophobic column chromatography and to identify the glyoxal-labeled peptide by scintillation counting. The experiment failed. These latter studies would have been facilitated by modern matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF MS), but unfortunately for the project, these were times well before the advent of modern mass spectroscopy.

Following his success with the band 3 system, Joe Falke moved on to the glucose transporter in the red cell ghost. There were many fewer copies of the glucose transporter in the red cell (10^4 copies of the glucose transporter versus 10^6 copies of the anion transporter), but glucose binding and transport were specific to this transporter. Thus, a substrate-binding assay was developed based on changes in the spectroscopy of the substrate when it became bound to the transporter. Joe and Jin Wang exploited 1H NMR nuclear Overhauser enhancement as an assay for glucose binding (103).

Particulate Methane Monooxygenase: A Membrane-Bound Enzyme that Oxidizes Methane to Methanol

During the early 1990s, Caltech was developing a program in C-H activation with the goal of designing a catalyst for the facile conversion of methane to methanol. As part of this effort, Professor Mary Lidstrom, a microbial geneticist working in the Division of Engineering and Applied Science at Caltech, and I assembled a team to try to understand how this chemistry was carried out by methanotrophic bacteria, which consumed methane produced in anaerobic sediments. These bacteria possess enzymes that catalyze the conversion of methane to methanol using dioxygen as a cosubstrate at ambient pressures and temperatures with high efficiency. The manner by which this conversion is accomplished in bacteria is of great interest to industrial chemists, as the controlled oxidation of methane to methanol is a difficult process in the laboratory, requiring expensive catalysts operating at high temperatures and pressures.

There are two of these enzymes: the membrane-bound monooxygenase (pMMO) and the soluble methane monooxygenase (sMMO), which are found in the cytoplasm of certain strains of bacteria. During the 1980s Professor John Lipscomb of the University of Minnesota isolated and purified sMMO to homogeneity (61). Accordingly, Mary Lidstrom and I decided to focus on the pMMO. Preliminary evidence for the existence of a membrane-bound methane monooxygenase was first reported by the laboratory of Professor Howard Dalton at the University of Warwick (82, 94). However, the pMMO had resisted both initial identification and subsequent isolation and purification for biochemical and biophysical characterization because of its instability outside the lipid bilayer and its tendency to lose essential metal cofactors during isolation and purification.

Mary Lidstrom was to focus on the molecular biology, and the Chan laboratory on the isolation, purification, and characterization of the pMMO from the membranes. Drew Schimke,

a postdoc, was recruited to initiate this effort. One of his first observations was that the pMMO was an abundant protein in the membranes of *Methylococcus capsulatus* (Bath) when this bacterium was cultured under high copper concentrations in the growth media. Accordingly, some of the properties of the enzyme could be inferred from physical characterization of the membranes obtained under these conditions.

Soon thereafter, Hoa Nguyen joined the pMMO project as a PhD student. Together with Drew, he carried out a detailed study of the effects of copper concentration on the methane monooxygenase activity of the membranes isolated from cells of *M. capsulatus*, and correlated the specific activity with the level of copper that was determined to be associated with these membranes. They concluded that the pMMO was indeed produced in high levels in the membranes when the bacterium was cultured at high copper concentration in the growth media, and that it was a multicopper protein containing as many as 15 copper ions per 100 kDa (71). From a quantitative proteomic analysis of bacterial cells obtained when the bacterium was grown under copper stress (48), we now know that copper ions are essential for the expression of the pMMO gene and growth of the organism. Not only do copper ions serve as a transcriptional activator of the pMMO gene, but they also enhance the expression of all the genes involved in cellular metabolism, including lipid biosynthesis. In addition, pMMO is a membrane protein containing high levels of copper ions, and these coppers are required for protein function (16).

Toward studying the enzyme in greater depth, Hoa succeeded in developing protocols to isolate and purify the enzyme to homogeneity despite numerous false starts (69). From sodium dodecyl sulfate gels of the subunits, Hoa obtained the N-terminal sequences of the subunits, information that was needed to fish out and clone the pMMO genes (90). Hoa also began to characterize both the pMMO-enriched membranes and the purified protein by EPR, X-ray Cu K α -edge absorption spectroscopy,

pMMO: particulate methane monooxygenase

sMMO: soluble methane monooxygenase

and SQUID magnetization measurements (69, 70). It was evident from these experiments that pMMO contained many copper ions and that some of these copper ions were sequestered into clusters.

In a groundbreaking experiment carried out in collaboration with Heinz Floss of the University of Washington and Philip Williams of the Tritium NMR Facility at the Lawrence Berkeley Laboratory, we showed that the hydroxylation of cryptically chiral ethane proceeded with total retention of configuration at the carbon center oxidized (107). This result suggested concerted oxenoid insertion across the C–H bond of the alkane, a reaction pathway distinct from the radical mechanism suggested for the nonheme diiron cluster in sMMO (61). Mei Zhu and Sean Elliott carried out further studies to clarify the regiospecificity and stereochemistry of the hydroxylation mediated by the pMMO, extending the substrates to include propane, butane, pentane, propene, and 1-butene (35). In propane, butane, and pentane, only the secondary alcohol was produced. Steve Yu synthesized deuterated chiral butanes and confirmed that the transfer of the O-atom to the secondary carbon occurs with total retention of configuration (112), in accord with a concerted oxo-insertion mechanism.

I retired from Caltech and moved to Academia Sinica in Taiwan in the fall of 1997. Despite my administrative responsibilities, initially as Director of the Institute of Chemistry, and subsequently as Vice President of Academia Sinica, I maintained an active research program. The conversion of methane to methanol by pMMO has been one of the research targets of the Chan laboratory in Taiwan during the past decade. During this period, Steve Yu, Kelvin Chen, Charlie Chen, and others developed methods to obtain large quantities of high-quality pMMO that were judged to be homogeneous according to subunit composition, complement of metal cofactors, and molecular mass (28, 111). Progress in our work on pMMO was also facilitated by major technological advances in the study of proteins since

our work on CcO some 30 years earlier, including high-throughput genome sequencing, bioinformatics, MALDI-TOF MS and ESI MS (electrospray ionization mass spectrometry) for proteomics and peptide mass fingerprinting, and ATR FTIR (attenuated total reflectance Fourier transform infrared spectroscopy) for secondary structure determination of proteins in membranes. Our progress toward understanding pMMO structure and function is just another example of how modern technology can fuel the advances in basic science.

In-depth studies have now revealed how the enzyme functions. It was necessary to carry out the structural and biophysical/biochemical studies on the enzyme with all the cofactors intact. In our work, a preparation of the enzyme with the full complement of 15 Cu ions was always used (26). At the active site of this enzyme is a trinuclear copper cluster (24, 26, 68) that, upon activation by molecular oxygen, mediates the transfer of a singlet oxene to the methane at the active site. A recent crystal structure of pMMO from *M. capsulatus* did not reveal a trinuclear copper center in the structure (60), but the protein preparation on which the crystal structure was based did not possess enzyme activity. The lesson we can all learn from this example is that protein isolation and purification must be carried out with adequate controls to ensure the biochemical integrity before we embark on biophysical and structural measurements. Those of us who conduct biophysical measurements are often less circumspect about the quality of the sample than they should be. Fortunately, in the case of pMMO, it was possible to reconcile the biochemical/biophysical data with the crystal structure by introducing the missing metallic cofactors back into the protein scaffold (24, 26, 68) (**Figure 3**).

Using a triad of copper ions to harness a singlet oxene offers a new mechanism for the controlled oxidation of an organic substrate (**Figure 4**). Analysis by density functional theory of various mechanistic scenarios by Peter Chen confirmed that the proposed direct singlet oxene insertion mediated by the trinuclear

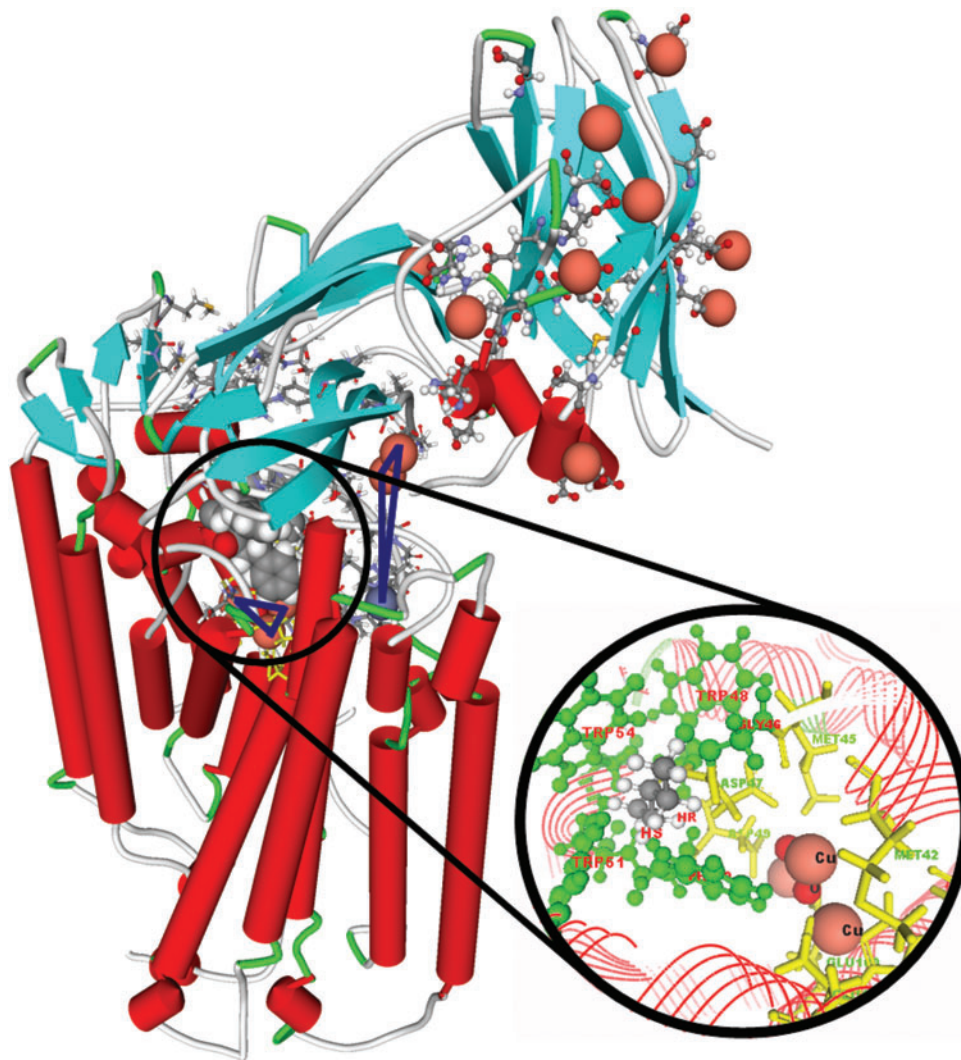


Figure 3

Structure of the particulate methane monooxygenase (pMMO) from *Methylococcus capsulatus* (Bath) with the active site, consisting of both the three-copper cluster and the hydrocarbon-binding pocket (*inset*). Figure taken from Reference 26.

copper cluster offered the most facile pathway for the alkane hydroxylation (29), and this mechanism yielded catalytic turnover rates and $^1\text{H}/^2\text{H}$ kinetic isotope effect values in close agreement with experiment.

The Chan laboratory is now exploiting the new chemistry toward developing a cheap and efficient catalyst for the controlled oxidation of methane and other small alkanes under ambi-

ent conditions of temperature and pressure. A model trinuclear copper cluster has been designed and synthesized to provide further support for the ideas derived from the enzyme studies. Our model trinuclear copper cluster can mediate facile oxo-insertion across C–C and C–H bonds in small organic substrates (30). The same chemistry seems to be operative here as in pMMO.

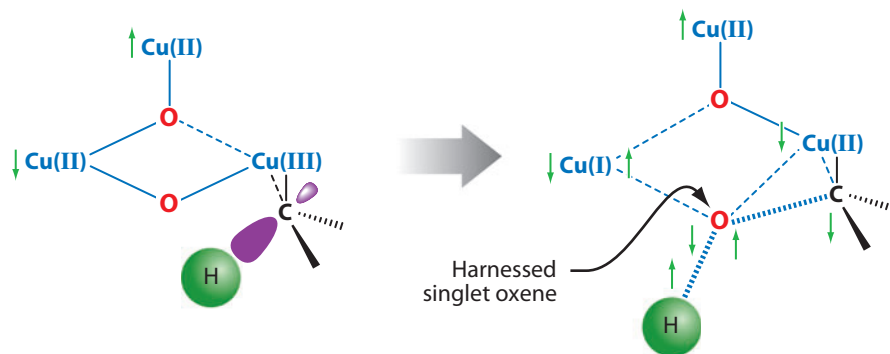


Figure 4

The singlet oxene mechanism for oxene insertion across a C-H bond from a dioxygen-activated trinuclear copper cluster to methane in pMMO. The harnessed singlet oxene is highlighted in the transition state. Figure adapted from Reference 29.

RETURN TO A SIMPLER ISSUE: EARLY KINETIC EVENTS IN PROTEIN FOLDING

To close out my scientific career, I decided to return to a simpler issue as well. Before the protein folding funnel of Peter Wolynes, José Onuchic, and David Thirumalai (108), I had begun to ask “Why do proteins fold so rapidly in water?” Small globular proteins fold into well-defined, three-dimensional structures with biological function quickly, usually within a few seconds or less. During the early 1990s, before I took early retirement from Caltech, I initiated another project that was subsequently carried over to Academia Sinica for continuation.

This work was premised on the hypothesis that the kinetic events that took place during the early stages of protein folding consisted of the formation of structural elements, and that the formation of the molten globule involved the hydrophobic collapse of secondary structures that were already preformed. To test this idea, Ron Rock and Kirk Hansen developed the photolabile caged-peptide strategy to measure the kinetics of the formation of different structural motifs (α -helices, β -sheets, hairpins). The method exploited a photolabile caged compound based on 3'-methoxybenzoin to cyclize the N terminus of the peptide to a side chain to disrupt the secondary structure (18, 42, 83). To trigger the refolding of the

peptide, the photolinker was cleaved rapidly (10^{-10} s) by a light pulse with good yield (quantum yield 0.6–0.7), and the refolding of the uncaged peptide was monitored by photoacoustic calorimetry. The method allowed us to observe the refolding of a structural element at times as early as 10^{-9} s and over a time span as long as 10^{-6} s. Another advantage was that the refolding process could be observed under ambient temperature and physiological pH without the interference from denaturants.

Using this approach, Rita Chen, Joseph Huang, Nicole Kuo, Howard Jan, Marappan Velusamy, Chung-Tien Lee, Wunshain Fann, Jaroslava Miksovska, and Randy Larsen measured the folding/refolding kinetics of specific structural motifs, including α -helices, hairpins, and β -sheets (31). These experiments revealed that these different structural motifs form quickly but, more surprisingly, according to a hierarchy of well-defined timescales, that is, a different time window for each type of structural motif. Moreover, the method was sufficiently sensitive, enabling them to observe subtle details in the protein folding (51).

Protein folding begins with a series of local folding processes driven by different interactions, and they are separated in time according to a highly ordered hierarchy. For example, when the folding process is driven by the local turn structure formation, the folding rate

is much faster than that driven by interstrand hydrophobic interaction. Thus, α -helices are formed first, followed by hairpins and β -sheets with strong turn-promoting sequences, which are in turn followed by hairpins and β -sheets that refold via nucleation by hydrophobic interactions. At the outset, local structural preference is derived from sequence steric hindrance, so the conformational space that needs to be sampled in the search is significantly reduced. These events would then be followed by further hydrophobic collapse of the remaining hydrophobic residues to form the molten globule and by subsequent annealing of the protein toward its global minimum. The global tertiary collapse needed to form the molten globule is expected to be slower than the refolding of hairpins via hydrophobic nucleation because the remaining hydrophobic residues must traverse over longer distances to displace the water molecules in the tertiary collapse. In the case of multidomain proteins, chaperones might be required to keep the domains apart spatially during this global hydrophobic collapse. On the other hand, for a protein with a relatively smooth free-energy landscape, the limit often referred to as minimal frustration, there would practically be no molten globule formed, and the structure would collapse to the native state once the topological fold is identified. Finally, the subsequent annealing of the protein is even slower yet, and a number of folding intermediates might be involved. The overall process is schematically illustrated in **Figure 5**, together with timescales expected for the various steps.

The early formation of the secondary structure or structural elements has been advocated by Baldwin, Englander, and others long ago. Their celebrated ideas were motivated mostly on thermodynamic grounds. In the end, folding pathways are dictated by kinetic considerations. In other words, protein folding occurs by kinetic channeling. As a kinetically controlled process, the protein follows a pathway of steepest descent in free energy within the protein folding funnel without sampling all points in conformational space in search of the na-

tive state. In this manner, Levinthal's paradox is avoided (59).

Because the information regarding the propensity of a polypeptide toward forming different structural motifs is built into the primary sequence of a protein, it follows that the details relating to protein folding are also encoded in the primary sequence of the polypeptide. This is a perfect example of how evolution has led to modularity of structural elements to allow a protein to fold in an orderly fashion in the otherwise highly complex free-energy landscape, as suggested by Michael Deem (99).

EPILOGUE

I have had an exciting career. The journey that ultimately led me to the world of membrane proteins was hardly programmed. It evolved serendipitously (logically though) from the chemical physics of simpler systems, from the dynamic structure of the lipid bilayer to lipid-protein interactions, to membrane biophysics of ion transport, and culminated in the structure and function of several membrane protein systems. I have taken advantage of my training in physical chemistry and modern spectroscopy and applied this knowledge to probe and solve interesting problems in modern biochemistry. As the systems became more complex, I learned how to manage the research undertaking, how to define a myriad of complex biological problems from scratch without knowing much about them, and how to formulate research strategies to approach the various issues. The complexity of these problems dictates that progress could only evolve, with the next generation of experiments building on the outcome of earlier ones. A problem like pMMO is so complex that it transcends generations of coworkers. In academia, and as a research mentor, I worked with students and postdocs as they joined my research group. With limited research resources, it is not possible to build a large team and solve problems in a short amount of time as is done in industry. However, with a more deliberate pace, it is possible to take advantage of emerging technologies as they

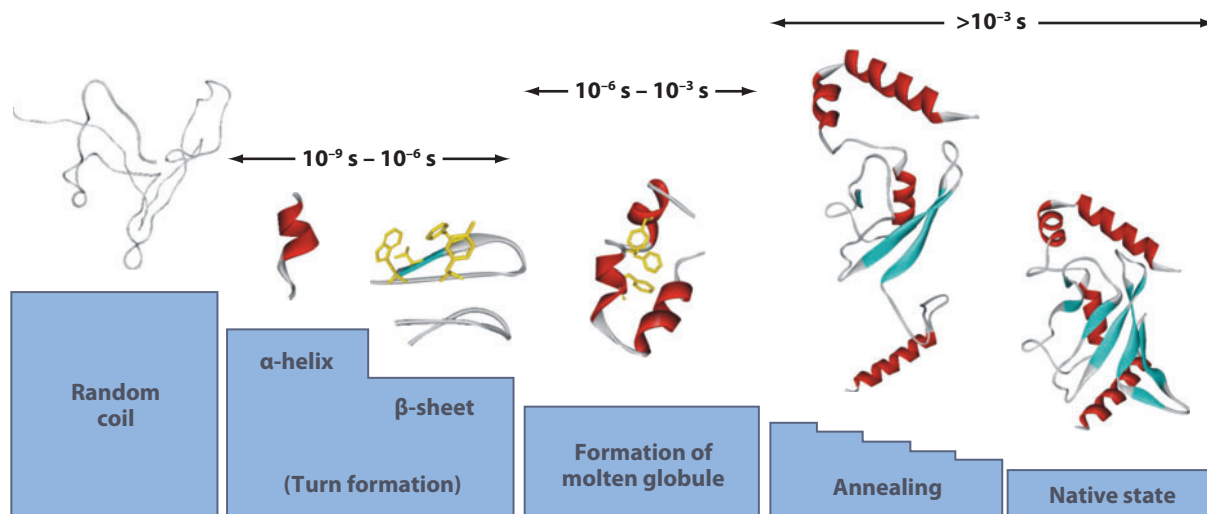


Figure 5

Kinetic hierarchy of the events in the folding of a single-domain protein, starting with the formation of the secondary structure to the formation of the molten globule, followed by thermal annealing of partially folded intermediates to reach the native structure.

develop. I have learned that technologies fuel the development of basic science, and a certain key technology can often make the difference.

The bulk of the membrane research accomplished at Caltech was supported by the National Institute of General Medical Sciences of NIH. Because my work on membranes evolved logically from model membranes to proteins in the cell membrane, I was able to sustain NIH funding for this project for more than 30 years. The level of support was adequate to cover most research expenditures, with the exception of upgrades of research instrumentation or the purchases of major pieces of equipment. After I moved to Taiwan, my research program was funded by Academia Sinica. Although the level of support was not as high as the NIH support I had at Caltech, Academia Sinica provided funds for upgrades of research facilities and the acquisition of state-of-the-art instrumentation as justified by the research. This capability greatly accelerated the progress of the scientific work I conducted in Taiwan.

I have learned much from my graduate students, postdocs, and colleagues. In addition, many people have opened doors for me and have contributed to the shaping of my scien-

tific career. Many of my colleagues at Caltech have been inspirational. As many as 200 graduate students, postdocs, and visitors (I have lost count) have passed through my laboratory. Unfortunately, this brief chapter does not allow me to mention each and every one.

Many of my former students and postdocs have gone on to do new science and even open new areas of research. I am proud of them. But that's the way it should be. In retrospect, I like to think that my style of training students works. The most important part of doing science is asking questions, and I taught my students to ask questions and how to ask questions. Without the questions, good or bad ones, there is no problem to solve. Discoveries in science are made by asking questions, from following up on leads, making observations in experiments, formulating hypotheses, and testing them by designing new experiments. The whole process from hypothesis to truth is often short-circuited by good intuition, which is, of course, based on knowledge or sound grounding in fundamentals, knowing what is reasonable and unreasonable in order to avert blind alleys.

Finally, I like to think that no matter how complicated biological problems may appear to

be, there is a simple answer to the outcome when all is said and done. In my view, nature is intrinsically simple, and it is fun to discover how the behavior of a complex biological system is explicable in terms of the laws of physics and chemistry as we now understand them to be.

DISCLOSURE STATEMENT

The author is not aware of any biases that might be perceived as affecting the objectivity of this review.

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LITERATURE CITED

1. Banerjee U, Zidovetzki R, Birge R, Chan SI. 1985. Interaction of alamethicin with lecithin bilayers: a ^{31}P and ^2H NMR study. *Biochemistry* 24:7621–27
2. Bangerter BW, Chan SI. 1968. A proton magnetic resonance study of the interaction of adenosine with polyuridylic acid: evidence for both adenine-uracil base-stacking and base-pairing. *Proc. Natl. Acad. Sci. USA* 60:1144–51
3. Bangerter BW, Chan SI. 1969. Proton magnetic resonance studies of ribose dinucleoside monophosphates in aqueous solution. The nature of the base-stacking interaction in adenylyl (3' 5') cytidine and cytidyl (3' 5') adenosine. *J. Am. Chem. Soc.* 91:3910–21
4. Beinert H, Griffiths DE, Wharton DC, Sands RH. 1962. Properties of the copper associated with cytochrome oxidase as studied by paramagnetic resonance spectroscopy. *J. Biol. Chem.* 237:2337–46
5. Blair DF, Gelles J, Chan SI. 1986. Redox-linked proton translocation in cytochrome oxidase: the importance of gating electron flow. *Biophys. J.* 50:713–33
6. Blair DF, Martin CT, Gelles J, Wang H, Brudvig GW, et al. 1983. The metal centers of cytochrome *c* oxidase: structures and interactions. *Chem. Scr.* 21:43–53
7. Blair DF, Witt SN, Chan SI. 1985. The mechanism of cytochrome *c* oxidase-catalyzed dioxygen reduction at low temperatures. Evidence for two intermediates at the three-electron level and entropic promotion of the bond-breaking step. *J. Am. Chem. Soc.* 107:7389–99
8. Bocian DF, Chan SI. 1978. NMR studies of membrane structure and dynamics. *Annu. Rev. Phys. Chem.* 29:307–35
9. Bull TE, Barthel JS, Jonas J. 1971. The effect of pressure on the F-19 spin-rotation interactions in benzotrifluoride in the liquid state. *J. Chem. Phys.* 54:3663–66
10. Burke TE, Chan SI. 1970. Nuclear spin relaxation in the presence of internal rotation. Nuclear-spin-internal-rotational coupling in benzotrifluoride and hexafluorobutylene-2. *J. Magn. Reson.* 2:120–40
11. Chan SI, Austin JA, Paez OA. 1969. Electron spin resonance studies of concentrated alkali metal-ammonia solutions. *Proc. Int. Union Pure Appl. Chem. Conf.*, pp. 425–38. New York: Cornell Univ. Press
12. Chan SI, Baker MR. 1961. Large rotational magnetic moment of NO. *Bull. Am. Phys. Soc.* 6:271
13. Chan SI, Baker MR, Ramsey NF. 1964. Molecular-beam magnetic resonance studies of the nitrogen molecule. *Phys. Rev.* 136:A1224–28
14. Chan SI, Bangerter BW, Peter HH. 1966. Purine binding to dinucleotides: evidence for base stacking and insertion. *Proc. Natl. Acad. Sci. USA* 55:720–27
15. Chan SI, Borgers TR, Russell JW, Strauss HL, Gwinn WD. 1966. Trimethylene oxide. Far-infrared spectrum and double-minimum vibration. *J. Chem. Phys.* 44:1103–11
16. Chan SI, Chen KHC, Yu SSF, Chen CL, Kuo SSJ. 2004. Toward delineating the structure and function of the particulate methane monooxygenase (pMMO) from methanotrophic bacteria. *Biochemistry* 43:4421–30

17. Chan SI, Feigenson GW, Seiter CHA. 1971. Nuclear relaxation studies of lecithin bilayers. *Nature* 231:110–12
18. Chan SI, Huang JJT, Larsen RW, Rock RS, Hansen KC. 2005. Early kinetic events in protein folding: the development and applications of caged peptides. In *Dynamic Studies in Biology: Phototriggers, Photoswitches and Caged Biomolecules*, ed. M Goeldner, R Givens, pp. 479–94. Weinheim, Ger.: Wiley-VCH Verlag GmbH
19. Chan SI, Kula RJ, Sawyer DT. 1964. The proton magnetic resonance spectra and structures of ethylenediaminetetraacetic acid, methyliminodiacetic acid, and nitrilotriacetic acid chelates of molybdenum. *J. Am. Chem. Soc.* 86:377–79
20. Chan SI, Li PM. 1990. Cytochrome *c* oxidase: understanding nature's design of a proton pump. *Biochemistry* 29:1–12
21. Chan SI, Nelson JH. 1969. Proton magnetic resonance studies of ribose dinucleoside monophosphates in aqueous solution. The nature of the base-stacking interaction in adenyl (3' 5') adenosine. *J. Am. Chem. Soc.* 91:168–83
22. Chan SI, Schweizer MP, Ts'o POP, Helmkamp GK. 1964. Interaction and association of bases and nucleosides in aqueous solutions. A nuclear magnetic resonance study of the self-association of purine and 6-methylpurine. *J. Am. Chem. Soc.* 86:4182–88
23. Chan SI, Seiter CHA, Feigenson GW. 1972. Anisotropic and restricted molecular motion in lecithin bilayers. *Biochem. Biophys. Res. Commun.* 46:1488–92
24. Chan SI, Wang VCC, Lai JCH, Yu SSF, Chen PPY, et al. 2007. Redox potentiometric studies of the particulate methane monooxygenase: support for a trinuclear copper cluster active site. *Angew. Chem. Int. Ed.* 46:1992–94
25. Chan SI, Witt SN, Blair DF. 1988. The dioxygen chemistry of cytochrome *c* oxidase. *Chem. Scr.* 28a:51–56
26. Chan SI, Yu SSF. 2008. Controlled oxidation of hydrocarbons by the membrane-bound methane monooxygenase: the case for a tricopper cluster. *Acc. Chem. Res.* 41:969–79
27. Chance B, Saronio C, Leigh JS. 1975. Functional intermediates in the reaction of membrane-bound cytochrome oxidase with oxygen. *J. Biol. Chem.* 250:9226–37
28. Chen KHC, Chen CL, Tseng CF, Yu SSF, Ke SC, et al. 2004. The copper clusters in the particulate methane monooxygenase (pMMO) from *Methylococcus capsulatus* (Bath). *J. Chin. Chem. Soc.* 51:1081–98
29. Chen PPY, Chan SI. 2006. Theoretical modeling of the hydroxylation of methane as mediated by the particulate methane monooxygenase. *J. Inorg. Biochem.* 100(4):801–9
30. Chen PPY, Yang RBG, Lee JCM, Chan SI. 2007. Facile O-atom insertion into C–C and C–H bonds by a trinuclear copper complex designed to harness a “singlet oxene.” *Proc. Natl. Acad. Sci. USA* 104:14570–75
31. Chen RP, Huang JJ, Chen HL, Jan H, Velusamy M, et al. 2004. Measuring the refolding of β -sheets with different turn sequences on a nanosecond timescale. *Proc. Natl. Acad. Sci. USA* 101:7305–10
32. Clore GM, Andreasson LE, Karlsson B, Aasa R, Malmström BG. 1980. Characterization of the intermediates in the reaction of mixed-valence state soluble cytochrome oxidase with oxygen at low temperatures by optical and electro-paramagnetic-resonance spectroscopy. *Biochem. J.* 185:155–67
33. Dubin AS, Chan SI. 1967. Nuclear spin internal-rotation coupling. *J. Chem. Phys.* 46:4533–35
34. Eigenberg KE, Chan SI. 1980. The effect of surface curvature on the head-group structure and phase transition properties of phospholipid bilayer vesicles. *Biochim. Biophys. Acta* 599:330–35
35. Elliott SJ, Zhu M, Nguyen HHT, Yip JHK, Chan SI. 1997. The regio- and stereoselectivity of particulate methane monooxygenase from *Methylococcus capsulatus* (Bath). *J. Am. Chem. Soc.* 119:9949–55
36. Falke JJ, Chan SI. 1985. Evidence that anion transport by band 3 proceeds via a ping-pong mechanism involving a single transport site. A ^{35}Cl NMR study. *J. Biol. Chem.* 260:9537–44
37. Falke JJ, Chan SI. 1986. Molecular mechanisms of band 3 inhibitors: transport site inhibitors. *Biochemistry* 25:7888–94
38. Falke JJ, Pace RJ, Chan SI. 1984. Chloride binding to the anion transport binding sites of band 3: a ^{35}Cl NMR study. *J. Biol. Chem.* 259:6472–80
39. Falke JJ, Pace RJ, Chan SI. 1984. Direct observation of the transmembrane recruitment of band 3 transport sites by competitive inhibitors: a ^{35}Cl NMR study. *J. Biol. Chem.* 259:6481–94
40. Feigenson GW, Chan SI. 1974. Nuclear magnetic relaxation behavior of lecithin multilayers. *J. Am. Chem. Soc.* 96:1312–19

41. Glaser M, Simpkins H, Singer SJ, Sheetz M, Chan SI. 1970. On the interactions of lipids and proteins in the red blood cell membrane. *Proc. Natl. Acad. Sci. USA* 65:721–28
42. Hansen KC, Rock RS, Larsen RW, Chan SI. 2000. A method for photoinitiating protein folding in a non-denaturing environment. *J. Am. Chem. Soc.* 122:11567–68
43. Hartzell CR, Beinert H. 1974. Components of cytochrome *c* oxidase detectable by EPR spectroscopy. *Biochim. Biophys. Acta* 368:318–38
44. Horwitz AF, Horsley WJ, Klein MP. 1972. Magnetic resonance studies on membrane and model membrane systems: proton magnetic relaxation rates in sonicated lecithin dispersions. *Proc. Natl. Acad. Sci. USA* 69:590–93
45. Hu VW, Chan SI, Brown GS. 1977. X-ray absorption edge studies on oxidized and reduced cytochrome *c* oxidase. *Proc. Natl. Acad. Sci. USA* 74:3821–25
46. Iwata S, Ostermeier C, Ludwig B, Michel H. 1995. Structure at 2.8 Å resolution of cytochrome *c* oxidase from *Paracoccus denitrificans*. *Nature* 376:660–69
47. Kainosho M, Kroon PA, Lawaczeck R, Petersen NO, Chan SI. 1978. Chain length dependence of the ¹H NMR relaxation rates in bilayer vesicles. *Chem. Phys. Lipids* 21:59–68
48. Kao WC, Chen YR, Yi EC, Lee H, Tian Q, et al. 2004. Quantitative proteomic analysis of metabolic regulation by copper in *M. capsulatus* (Bath). *J. Biol. Chem.* 279:51554–60
49. Kroon PA, Kainosho M, Chan SI. 1975. The state of molecular motion of cholesterol in lecithin bilayers. *Nature* 256:582–84
50. Kroon PA, Kreishman GP, Nelson JH, Chan SI. 1974. The effects of chain length on the secondary structure of oligoadenylates. *Biopolymers* 13:2571–92
51. Kuo NNW, Huang JJT, Miksovská J, Chen RPY, Larsen RW, et al. 2005. Effects of turn mutation on the structure, stability, and folding kinetics of a β-sheet peptide. *J. Am. Chem. Soc.* 127:16945–54
52. Kyogoku Y, Lord RC, Rich A. 1966. Hydrogen bonding specificity of nucleic acid purines and pyrimidines in solution. *Science* 154:518–20
53. Kyogoku Y, Lord RC, Rich A. 1967. An infrared study of hydrogen bonding between adenine and uracil derivatives in chloroform solution. *J. Am. Chem. Soc.* 89:496–504
54. Lau ALY, Chan SI. 1974. Nuclear magnetic resonance studies of the interaction of alamethicin with lecithin bilayers. *Biochemistry* 13:4942–48
55. Lau ALY, Chan SI. 1975. Alamethicin-mediated fusion of lecithin vesicles. *Proc. Natl. Acad. Sci. USA* 72:2170–74
56. Lawaczeck R, Kainosho M, Chan SI. 1976. The formation and annealing of structural defects in lipid bilayer vesicles. *Biochim. Biophys. Acta* 433:313–30
57. Lawaczeck R, Kainosho M, Girardet JL, Chan SI. 1975. Effects of structural defects in sonicated phospholipid vesicles on fusion and ion permeability. *Nature* 256:584–86
58. Levine YK, Birdsall NJM, Lee AG, Metcalfe JC. 1972. Carbon-13 nuclear magnetic resonance relaxation measurements of synthetic lecithins and the effect of spin-labeled lipids. *Biochemistry* 11:1416–21
59. Levinthal C. 1968. Are there pathways for protein folding? *J. Chem. Phys.* 85:44–45
60. Lieberman RL, Rosenzweig AC. 2005. Crystal structure of a membrane-bound metalloenzyme that catalyzes the biological oxidation of methane. *Nature* 434:177–82
61. Lipscomb JD. 1994. Biochemistry of the soluble methane monooxygenase. *Annu. Rev. Microbiol.* 48:371–99
62. Marcus RA, Sutin N. 1985. Electron transfers in chemistry and biology. *Biochim. Biophys. Acta* 811:265–322
63. Martin CT, Scholes CP, Chan SI. 1988. On the nature of cysteine coordination to Cu_A in cytochrome *c* oxidase. *J. Biol. Chem.* 263:8420–29
64. Morgan JE, Li PM, Jang DJ, El-Sayed MA, Chan SI. 1989. Electron transfer between cytochrome *a* and copper A in cytochrome *c* oxidase: a perturbed equilibrium study. *Biochemistry* 28:6975–83
65. Mueller L, Chan SI. 1983. Two-dimensional deuterium NMR of lipid membranes. *J. Chem. Phys.* 78:4341–48
66. Musser SM, Chan SI. 1995. Understanding the cytochrome *c* oxidase proton pump: thermodynamics of redox linkage. *Biophys. J.* 68:2543–55

67. Musser SM, Stowell MHB, Chan SI. 1995. Cytochrome *c* oxidase: chemistry of a molecular machine. *Adv. Enzymol. Relat. Areas Mol. Biol.* 71:79–208
68. Ng KY, Tu LC, Wang YS, Chan SI, Yu SS. 2008. Probing the hydrophobic pocket of the active site in the particulate methane monooxygenase (pMMO) from *Methylococcus capsulatus* (Bath) by variable stereo-selective alkane hydroxylation and olefin epoxidation. *Chembiochem* 9:1116–23
69. Nguyen HHT, Elliott SJ, Yip JHK, Chan SI. 1998. The particulate methane monooxygenase from *Methylococcus capsulatus* (Bath) is a novel copper-containing three-subunit enzyme: isolation and characterization. *J. Biol. Chem.* 273:7957–66
70. Nguyen HHT, Nakagawa KH, Hedman B, Elliott SJ, Lidstrom ME, et al. 1996. X-ray absorption and EPR studies on the copper ions associated with the particulate methane monooxygenase from *Methylococcus capsulatus* (Bath). Cu(I) ions and their implications. *J. Am. Chem. Soc.* 118:12766–76
71. Nguyen HHT, Shiemke AK, Jacobs SJ, Hales BJ, Lidstrom ME, et al. 1994. The nature of the copper ions in the membranes containing the particulate methane monooxygenase from *Methylococcus capsulatus* (Bath). *J. Biol. Chem.* 269:14995–5005
72. Owicki JC, McConnell HM. 1979. Theory of protein-lipid and protein-protein interactions in bilayer membranes. *Proc. Natl. Acad. Sci. USA* 76:4750–54
73. Owicki JC, Springgate MW, McConnell HM. 1978. Theoretical study of protein-lipid interactions in bilayer membranes. *Proc. Natl. Acad. Sci. USA* 75:1616–19
74. Pace RJ, Chan SI. 1982. Molecular motions in lipid bilayers. II. Magnetic resonance of multilamellar and vesicle systems. *J. Chem. Phys.* 76:4228–40
75. Pearson LT, Chan SI. 1982. Effects of lipid-mediated interactions on protein pair distribution functions. *Biophys. J.* 37:141–42
76. Pearson LT, Chan SI. 1987. Pair distribution functions of cytochrome *c* oxidase in lipid bilayers: evidence for a lipid-mediated repulsion between protein particles. *Chem. Scr.* 27B:203–9
77. Pearson LT, Edelman J, Chan SI. 1984. Statistical mechanics of lipid membranes: protein correlation functions and lipid ordering. *Biophys. J.* 45:863–71
78. Pearson LT, Lewis BA, Engleman DM, Chan SI. 1983. Pair distribution functions of bacteriorhodopsin and rhodopsin in model bilayers. *Biophys. J.* 43:167–74
79. Petersen NO, Chan SI. 1977. More on the motional state of lipid bilayer membranes. The interpretation of order parameters obtained from nuclear magnetic resonance experiments. *Biochemistry* 16:2657–67
80. Prestegard JH, Chan SI. 1969. Proton magnetic resonance studies of the cation-binding properties of nonactin. The K⁺-nonactin complex. *Biochemistry* 8: 3921–27
81. Prestegard JH, Chan SI. 1970. Proton magnetic resonance studies of the cation-binding properties on nonactin. Comparison of the sodium ion, potassium ion, and cesium ion complexes. *J. Am. Chem. Soc.* 92:4440–46
82. Prior SD, Dalton H. 1985. The effect of copper ions in membrane content and methane monooxygenase activity in methanol-grown cells of *Methylococcus capsulatus* (Bath). *J. Gen. Microbiol.* 131:155–63
83. Rock RS, Chan SI. 1998. Preparation of a water-soluble “cage” based on 3',5'-dimethoxybenzoin. *J. Am. Chem. Soc.* 120:10766–67
84. Schmidt CF Jr, Chan SI. 1971. Nuclear spin-lattice relaxation of the methyl carbon-13 in toluene. *J. Magn. Reson.* 5:151–54
85. Schuh J, Banerjee U, Mueller L, Chan SI. 1982. The phospholipid packing arrangement in small bilayer vesicles as revealed by proton magnetic resonance studies at 500 MHz. *Biochim. Biophys. Acta* 687:219–25
86. Schultz BE, Chan SI. 2001. Structures and proton-pumping strategies of mitochondrial respiratory enzymes. *Annu. Rev. Biophys. Biomol. Struct.* 30:23–65
87. Schweizer MP, Chan SI, Helmkamp GK, Ts'o POP. 1964. An experimental assignment of the proton magnetic resonance spectrum of purine. *J. Am. Chem. Soc.* 86:696–700
88. Seelig J, Seelig A. 1974. Dynamic structure of fatty acyl chains in a phospholipid bilayer measured by deuterium magnetic resonance. *Biochemistry* 13:4839–45
89. Seiter CHA, Chan SI. 1973. Molecular motion in lipid bilayers: a nuclear magnetic resonance linewidth study. *J. Am. Chem. Soc.* 95:7541–53
90. Semrau J, Chistoserdov A, Lebron J, Costello A, Davagnino J, et al. 1995. Particulate methane monooxygenase genes in methanotrophs. *J. Bacteriol.* 177:3071–79

91. Sheetz MP, Chan SI. 1972. Effect of sonication on the structure of lecithin bilayers. *Biochemistry* 11:4573–81
92. Sheetz MP, Chan SI. 1972. Proton magnetic resonance studies of whole human erythrocyte membranes. *Biochemistry* 11:548–55
93. Singer SJ, Nicolson GL. 1972. The fluid mosaic model of the structure of cell membranes. *Science* 175:720–31
94. Smith DDS, Dalton H. 1989. Solubilization of methane monooxygenase from *Methylococcus capsulatus* Bath. *Eur. J. Biochem.* 182:667–72
95. Stevens TH, Brudvig GW, Bocian DF, Chan SI. 1979. The structure of the cytochrome a_3 -Cu $_3$ couple in cytochrome c oxidase as revealed by nitric oxide binding studies. *Proc. Natl. Acad. Sci. USA* 76:3320–24
96. Stevens TH, Chan SI. 1981. Histidine is the axial ligand to cytochrome a_3 in cytochrome c oxidase. *J. Biol. Chem.* 256:1069–71
97. Stevens TH, Martin CT, Wang H, Brudvig GW, Scholes CP, et al. 1982. The nature of Cu $_3$ in cytochrome c oxidase. *J. Biol. Chem.* 257:12106–13
98. Stockton G, Polnaszek CF, Tulloch AP, Hasan F, Smith ICP. 1976. Molecular motion and order in single-bilayer vesicles with multilamellar dispersions of egg lecithin and lecithin-cholesterol mixtures. A deuterium nuclear magnetic resonance study of specifically labeled lipids. *Biochemistry* 15:954–66
99. Sun J, Deem MW. 2007. Spontaneous emergence of modularity in a model of evolving individuals. *Phys. Rev. Lett.* 99:228107(4)
100. Ts'o POP, Chan SI. 1964. Interaction and association of bases and nucleosides in aqueous solutions. Association of 6-methylpurine and 5-bromouridine and treatment of multiple equilibria. *J. Am. Chem. Soc.* 86:4176–81
101. Tsukihara T, Aoyama H, Yamashita E, Tomizaki T, Yamaguchi H, et al. 1995. Structures of metal sites of oxidized bovine heart cytochrome c oxidase at 2.8 Å. *Science* 269:1069–74
102. Tsukihara T, Aoyama H, Yamashita E, Tomizaki T, Yamaguchi H, et al. 1996. The whole structure of the 13-subunit oxidized cytochrome c oxidase at 2.8 Å. *Science* 272:1136–44
103. Wang JF, Falke JJ, Chan SI. 1986. A proton NMR study of the mechanism of the erythrocyte glucose transporter. *Proc. Natl. Acad. Sci. USA* 83:3277–81
104. Watnick PI, Chan SI, Dea P. 1990. Hydrophobic mismatch in gramicidin A'/lecithin systems. *Biochemistry* 29:6215–21
105. Wikström M. 1989. Identification of the electron transfers in cytochrome oxidase that are coupled to proton-pumping. *Nature* 338:776–78
106. Wikström MK. 1977. Proton pump coupled to cytochrome c oxidase in mitochondria. *Nature* 266:271–73
107. Wilkinson B, Zhu M, Priestley ND, Nguyen HHT, Morimoto H, et al. 1996. A concerted mechanism for ethane hydroxylation by the particulate methane monooxygenase from *Methylococcus capsulatus* (Bath). *J. Am. Chem. Soc.* 118:921–22
108. Wolynes PG, Onuchic JN, Thirumalai D. 1995. Navigating the folding routes. *Science* 267:1619–20
109. Yoshikawa S, Tera T, Takahashi Y, Caughey WS. 1988. Crystalline cytochrome c oxidase of bovine heart mitochondrial membranes: composition and X-ray diffraction studies. *Proc. Nat. Acad. Sci. USA* 85:1354–58
110. Yu C, Yu L, King TE. 1975. Studies on cytochrome oxidase. Interactions of the cytochrome oxidase protein with phospholipids and cytochrome c . *J. Biol. Chem.* 250:1383–92
111. Yu SSF, Chen KHC, Tseng MYH, Wang YS, Tseng CF, et al. 2003. Production of high quality pMMO in high yields from *Methylococcus capsulatus* (Bath) with a hollow-fiber membrane bioreactor. *J. Bacteriol.* 185:5915–24
112. Yu SSF, Wu LY, Chen KHC, Luo WI, Huang DS, et al. 2003. The stereospecific hydroxylation of [2,2- 2 H $_2$]butane and chiral dideuteriobutanes by the particulate methane monooxygenase from *Methylococcus capsulatus* (Bath). *J. Biol. Chem.* 278:40658–69
113. Zaslavsky D, Sadoski RC, Wang K, Durham B, Gennis RB, et al. 1998. Single electron reduction of cytochrome c oxidase compound F: resolution of partial steps by transient spectroscopy. *Biochemistry* 37:14910–16