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Adventures in Bioinorganic Chemistry

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Prologue

Preparing an acceptance talk for the ACS Award for Distinguished Service in the Advancement of Inorganic Chemistry provided me the opportunity to reflect on the human side of my professional journey. Subsequently, I accepted the Editor's kind invitation to publish these reflections, in the hope that they will amuse my colleagues and that they may even hold some meaning for those starting out on their careers.

From Chemistry Set to Scandinavia

I liked chemistry from the moment my parents bought me a chemistry set when I was 12. I had a great time dissolving and mixing the metal salts, watching the resulting colors, and smelling the smells. My little sister was terrified that I would blow up the house, but somehow we survived. This introduction to chemistry was reinforced by Mr. Nicklin, my enthusiastic high school chemistry teacher. So, when I enrolled at the University of California at Los Angeles (UCLA), I majored in chemistry, though I did cast about in social science courses. I also considered a career in science journalism, after writing for the college paper, the *UCLA Daily Bruin*, and serving as its managing editor. However, the chemistry faculty were very supportive, and I decided to continue toward the Ph.D.

My favorite undergrad courses had been in analytical chemistry. So, when I arrived at the Massachusetts Institute of Technology (MIT) for graduate work, in 1956, I joined the analytical division, headed by David Hume and Lockhart (Buck) Rogers. Hume was a great thesis advisor. He left me to do pretty much what I wanted but was always available with thoughtful advice.

Research in the Hume laboratory focused on the measurement of metal complex formation constants. I chose mixed-halide complexes of mercury[1,2] for my thesis. Outside the laboratory, I took key courses in vibrational spectroscopy from Dick Lord and in group theory from Al Cotton, then a young turk on the faculty. Cotton's course gave me my first taste of the hot new field of ligand-field theory. When I finished my thesis, I won a Fulbright scholarship to study in Denmark. Hume and Cotton helped to get me a spot with Carl Ballhausen, who had just returned from Harvard

Thomas G. Spiro was the Eugene Higgins Professor of Chemistry at Princeton University, and former chair of the Chemistry Department, before relocating to the University of Washington (UW) in 2007. There he enjoys frequent access to twin 2-year-old grandsons, living in Seattle, and the stimulation of new colleagues at UW. Spiro's research program focuses on the roles of metal ions in biology and the application of vibrational spectroscopy to problems in biomolecular structure and dynamics. He has also been involved in research and teaching in environmental chemistry. He received a B.S. from UCLA and a Ph.D. from MIT (with David Hume) and did postdoctoral work in Copenhagen (with Carl Ballhausen) and Stockhom (with Lars Gunnar Sillin), before joining the Princeton University faculty in 1963. In 2004, Spiro received the ACS Award for Distinguished Service in the Advancement of Inorganic Chemistry and the Biophysical Society Founders Award. He was the Wellcome Visiting Professor in the Basic Medical Sciences, at the University of British Columbia, in 1999 and received the first Bomem−Michelson Award in Molecular Spectroscopy in 1986.

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University to become the youngest full professor in the Danish academic world.

In 1960, I set sail for Copenhagen with my bride of 1 year, Helen Handin (one still crossed the Atlantic by ship in those days). When Ballhausen showed me to my desk, my new office mate, also just arrived from the U.S., extended his hand and said, "Hi, I'm Harry Gray". And so began a lifelong friendship.

Harry and I read Ballhausen's classic book *Ligand Field Theory* in manuscript, and Harry went on to apply the theory to a host of interesting compounds. However, after making a trigonal nickel(II) complex and analyzing its spectrum,[3] I returned to my roots in complexation constants. Following 1 year of working for Standard Oil in Southern California, where I could be near my cancer-stricken father, I returned to Scandinavia, this time to the Stockholm laboratory of Lars Gunnar Sillen, the capital of complex formation measurements at the time. There I determined hydrolysis constants for thallium(III) chloride complexes.[4]

Princeton and Raman

From Stockholm, I landed a job at Princeton University. Helen and I moved there in 1963, and our sons Peter and Michael were born soon after. I was hired as an instructor in analytical chemistry. Princeton University had a distinguished analytical tradition, established by Howell Furman and Clark Bricker, but lost interest in analytical chemistry in the 1960s, as did many other schools. Luckily for me, I could by then pass as an inorganic chemist.

The thallium(III) chloride complexes I had been studying in Stockholm had irregular stability constants, and I was curious about their structures. I remembered from Lord's course at MIT that Raman spectroscopy was a useful structural tool in aqueous solution. Donald Hornig, my department chair, had just gone to Washington as Lyndon Johnson's science advisor and left behind a Cary 81 Raman spectrometer, in the care of his graduate student Steve Kittleberger. The Cary 81 was the pre-laser Cadillac of Raman spectroscopy and occupied a 5-ft-high metal cabinet (Figure 1). Steve showed me how to load my thallium(III) chloride solution into a horn-shaped tube, slip it horizontally into the coils of the mercury arc lamp, and then light the lamp with a Tesla coil. His jaw dropped as a beautiful spectrum came rolling out of the strip-chart recorder (Figure 1).[5] I had inadvertently chosen one of the strongest Raman scatterers, while he had been toiling away at one of the weakest: solid HF.

This experience hooked me on Raman spectroscopy. My first graduate students took advantage of the Hornig instrument to explore a number of metal complexes, including the polynuclear hydroxy complexes of bismuth(III),[6] lead(II),[7] and trimethylplatinum(IV),[8] with which I had become familiar in Stockholm. Their spectra gave evidence of interaction among the metal ions, leading us to explore the vibrational spectroscopy of metal−metal bonds in a series of metal cluster compounds.[9−13] Raman spectroscopy was well suited to this application because of the low frequencies and large polarizability derivatives of the metal−metal stretching vibratons.[14]

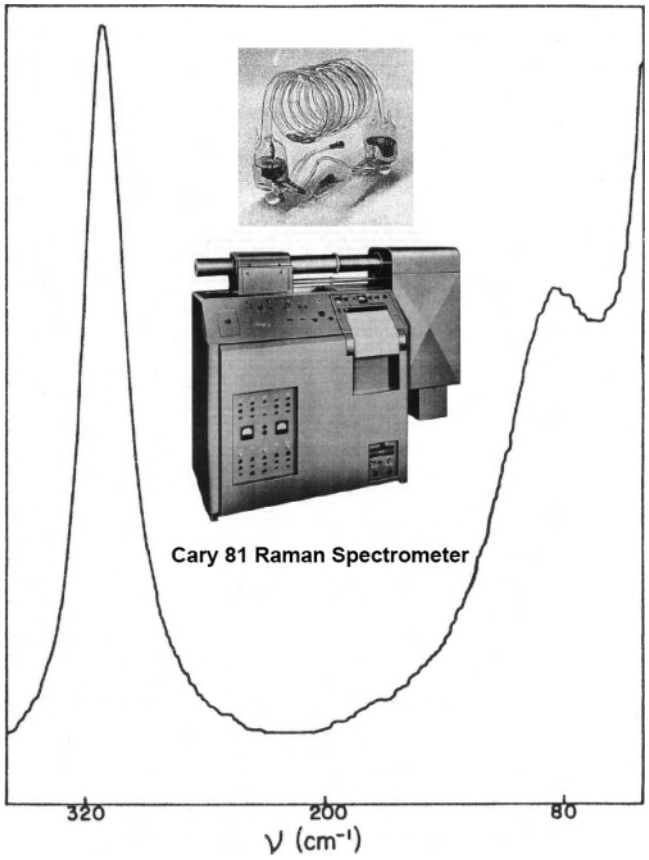

Figure 1. Raman spectrum of 0.9 M $TlCl_3$ in 1 M HCl and the Carey 81 Raman spectrometer with its mercury arc light source.[5]

Iron Balls and Environmental Chemistry

My stay in Stockholm had been financed by a NIH postdoctoral fellowship, which I had won with an application that argued the need for understanding the nature of metal complexes in biological systems. My arguments at the time were naïve, but this topic did, in fact, become the main theme of my research career. To find out more, I attended the Gordon Conference on "Metals in Biology" in 1964, in New Hampshire. On the first afternoon, I went for a swim in Squam Lake, and a big guy pulled himself up onto the raft beside me, stuck out his hand, and said "Hi, I'm Paul Saltman". Thus began another lifelong friendship.

This was only the second "Metals in Biology" conference, and coherence was not its strong point. Paul characterized it as the "EPR spectroscopists" talking with the "pig feeders", i.e., nutritionists trying to make sense of the roles of trace metals in the diet. Paul was one of the latter. Upon discovering that I knew something about metal complexes, he thrust a paper in my hand that showed that ferric salts in the presence of fructose were efficiently absorbed through

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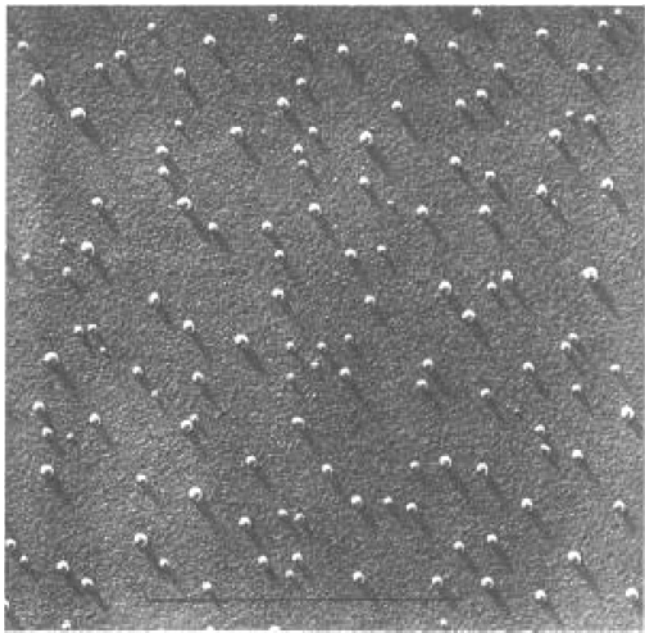

Figure 2. 70 Å ferric hydroxide particles obtained by HCO_3^- addition to $Fe(NO_3)_3$.[15] The line marks 1 μm.

the intestines. At the time, only ferrous salts were thought to be taken up.

Paul wanted me to tell him what the ferric fructose complex was. Fresh from my stay in Stockholm, I advised him to measure the formation curve via potentiometry. He looked doubtful but said "If you're so smart, come to my lab and do it yourself". I accepted the invitation, and next summer I set off for the Saltman laboratory at the University of Southern California (USC), which provided a nice opportunity to introduce 8-month-old Peter to both sets of grandparents in Los Angeles. We set up potentiometers, prepared electrodes and solutions, adjusted the pH, and waited for the potentials to settle down. However, they never did. They drifted for hours, even days. We had rediscovered ferric hydroxide colloid formation! The same chocolate brown solutions were formed if we left out the fructose and just neutralized ferric salts with bicarbonate. What made these colloids interesting, however, was that electron microscopy revealed a fairly uniform distribution of spherical particles, 70 Å across (Figure 2).[15] This was the same size as the hydroxyiron core of the iron storage protein ferritin.

Could this be a coincidence? We suggested that ferritin might form by templated assembly of the protein subunits around a naturally formed hydroxyiron core. This hypothesis is almost certainly incorrect. The protein shell is very stable, and one can load and unload iron into it, most readily via oxidation and reduction. Still, our work stimulated a good deal of research on what Harry dubbed "Saltman's balls". The physiological mechanisms of iron uptake and release are still being worked out.

I spent several enjoyable summers in the Saltman laboratory, first at USC and later at the University of California at San Diego, working on ferric hydroxide colloid stabilization.[16] Recently, my interest in metal oxides has been revived by a fruitful environmental science collaboration on the remarkable propensity of many bacteria to coat themselves with nanoparticulate MnO_2, produced by manganese(II) oxidation. We are working on the mechanism of this process, which appears to involve a multicopper oxidase.[17]

My interest in environmental science began in the politically turbulent 1970s when my department chair asked if I could perhaps teach a course that was "relevant". The first Earth Day materialized, and issues like DDT, global warming, and threats to the ozone layer began to seep into the news. I advertised a course in environmental chemistry and invited the 20 or so enrollees to accompany me to the library and research the background of these subjects. The course attracted the interest of Bill Stigliani, then a postdoc in the department, who volunteered to help teach it. Bill went on to a career in environmental science, and he and I eventually coauthored a textbook, *Chemistry of the Environment*.[18] I also became involved with environmental studies university-wide and helped to establish the Princeton Environmental Institute, which now includes the Center for Environmental Bioinorganic Chemistry.

Romance of Raman

Soon after arriving at Princeton University, I obtained a NIH grant to study biologically interesting complexes using Raman spectroscopy. The grant included $20K to purchase a new Raman light source, a 20-mW helium−neon laser that was being marketed as an attachment to the Cary 81. Fortunately, faculty colleague George Leroi persuaded me that this was not a good buy and that we should pool our resources to build a proper laser-Raman spectrometer, using the recently published design of Sergio Porto. This design, soon marketed by the Spex Corp., quickly swept the field, making the Cary 81 obsolete.

George and his students put together a spectrometer that worked well. The Porto design, taking advantage of the laser's directional property, allowed us to examine colored samples without losing all of the light to absorption. This capability, and the availability of several visible wavelengths from the Ar^+/Kr^+ mixed-gas laser that we bought, let us explore the resonance Raman (RR) effect, which amplifies the Raman cross section via coupling of vibrational and electronic transitions. This effect promised to greatly extend the utility of Raman spectroscopy, which normally required concentrated solutions because of low cross sections. There had been very few experimental studies of resonance enhancement because of the prelaser difficulties associated with colored samples. We felt like explorers on a new continent. Not only could we obtain RR spectra, but we could also study colored metalloproteins because the spectrum was dominated by vibrational bands of the chromophore; there was no interference from the myriad vibrations of the protein itself, which were unenhanced.

The quest was led by an early group of talented postdocs. One of the first was Jim Nestor, a master experimentalist who really got our instruments working and then trained the rest of the group in their proper use. He built an early CARS (coherent anti-Stokes Raman spectroscopy) instrument from

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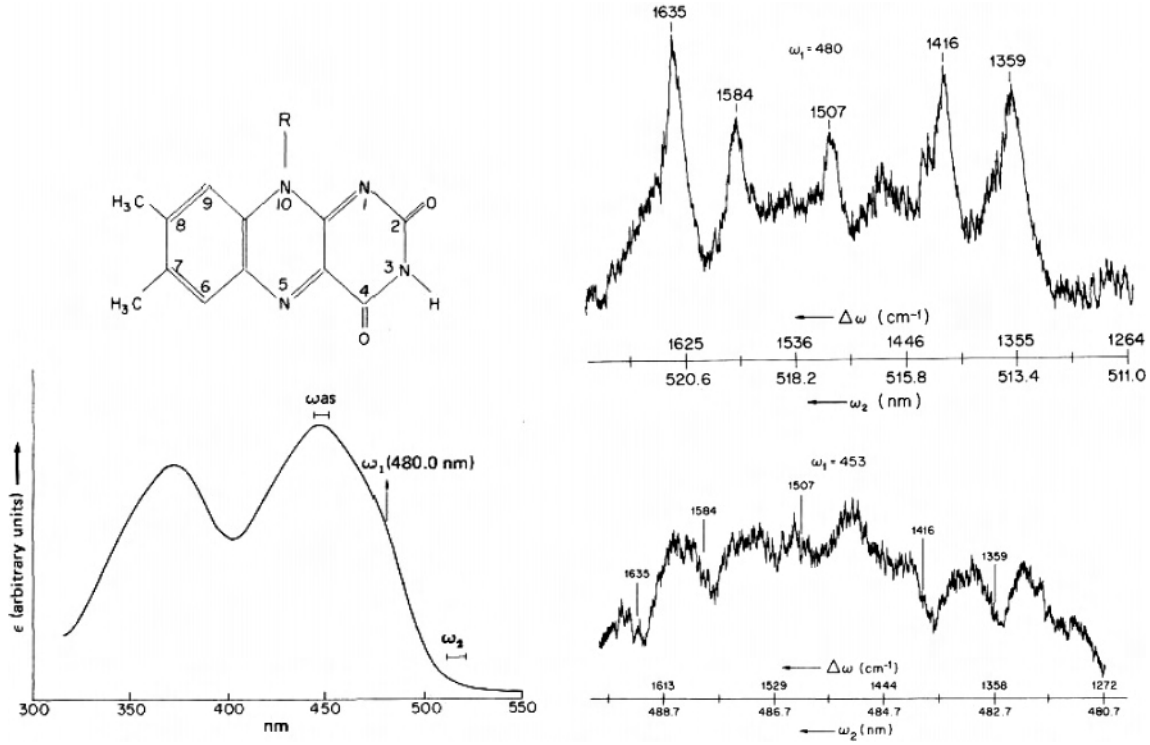

Figure 3. (Left) Flavin structure and absorption spectrum. (Right) CARS spectra, at two probe wavelengths, of the flavin cofactor in glucose oxidase, illustrating the complex CARS line shapes.[20] ω_1 is the fixed laser wavelength (nm), ω_2 is the scanned laser wavelength (nm), $\Delta\omega = \omega_1 - \omega_2$ (cm^{-1}), and $\omega_{as} = 2\omega_1 - \omega_2$ is the wavelength of the coherent beam generated in the medium from ω_1 and ω_2.

scratch, publishing landmark papers on the application of resonance CARS.[19,20] The appeal of CARS was that the directional character of its signal eliminates interference from fluorescence, which is the great bane of Raman spectroscopy. The catch was that the CARS line shapes are severely distorted (Figure 3) because of interference from an electronic background signal. Spectral interpretation required a complex analysis.[20] We abandoned the technique as too unwieldy, although others have persevered, and the advent of femtosecond techniques has given great impetus to CARS.

We were joined by Paul Stein, a talented theorist. Paul guided us through the interpretation of interesting phenomena that we were discovering, such as resonance deenhancement[21] and antisymmetric Raman scattering.[22−24]

Vinny Mizkowski joined us from Harry's laboratory. Vinny had an incredible nose for inorganic spectroscopy. He correctly deduced that the intense blue color of "blue" copper proteins and the complex RR spectrum they produced require a trigonal coordination geometry, with an equatorial cysteinate ligand, and weak axial ligation (Figure 4).[25] This work preceded the first crystal structure, by Hans Freeman, and the elegant spectroscopic analysis by Harry and by Ed Solomon.

We explored many-colored metalloproteins. In addition to the "blue" copper proteins, we studied the type III dicopper site, combining RR spectroscopy with extended X-ray absorption fine structure analysis, a technique then just emerging at the Stanford synchrotron.[26−29]

Iron−sulfur proteins became a major focus. Greg Kubas, one of my first postdocs, took a stab at preparing a model compound, which turned out to have the nonbiological but very interesting structure shown in Figure 5.[30] Later, when Dick Holm showed how to prepare proper models with sulfide and thiolate ligation, we compared their RR spectra with those of the proteins being modeled to decipher protein-specific effects. Roman Czernuszewicz and Mike Johnson led this effort.[31−34]

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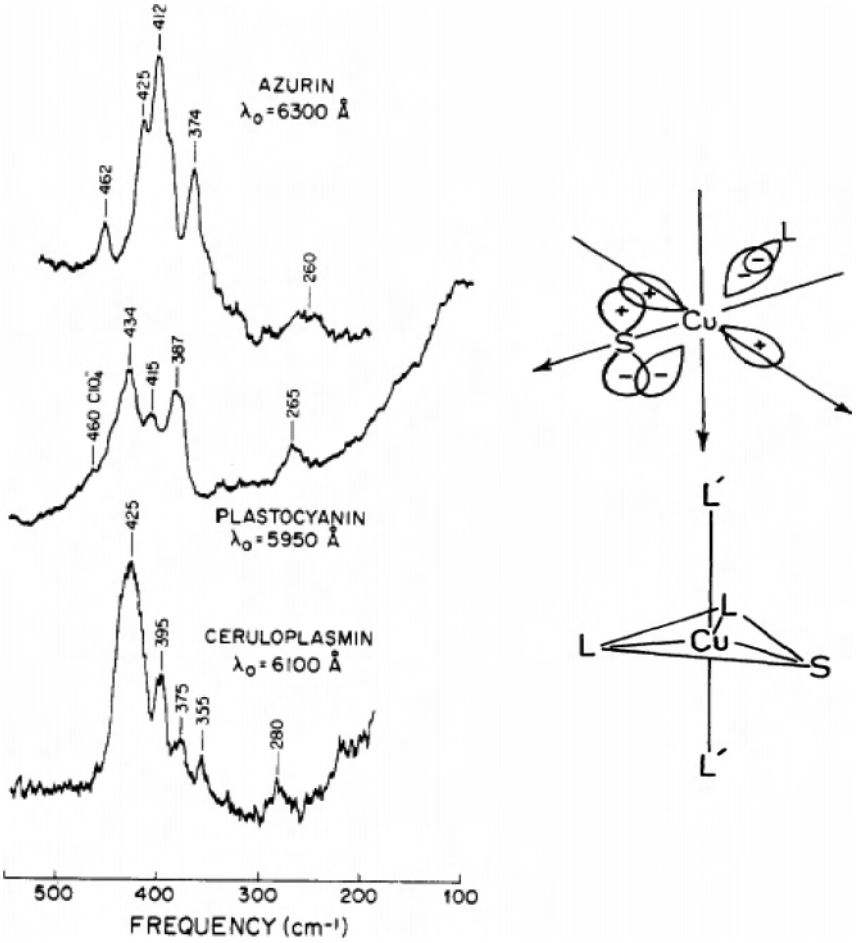

Figure 4. RR spectra of three "blue" copper proteins and Mizkowski's model of the active site.[18]

We studied cobalamins, as models for vitamin-B_{12}-containing enzymes.[35,36] In the early work, photolysis from the Raman laser precluded studies of the physiologically relevant alkylcobalamins, but later we discovered how to circumvent this problem using frozen solutions, leading to mechanistic studies of important enzymes.[37,38] There was a brief foray as well into molybdenum enzymes.[39,40]

Eventually we went beyond metal centers and investigated other biological chromophores, including flavins,[41,42] nicotinamide adenine dinucleotide,[43] and pyridoxal,[44] using various stratagems to circumvent fluorescence.

In a collaboration with biochemistry colleague Charles Gilvarg, we published the first Raman spectrum of a bacterial spore, which was dominated by the spore coat constituent calcium dipicolinate.[45] This spectrum has recently become the basis for promising approaches to detecting anthrax spores.

It was obvious that even bigger game lay in the ultraviolet region. Taking up the challenge, Rich Rava and Steve Fodor built an ultraviolet Raman (UVRR) spectrometer, based on a pulsed Nd:YAG laser, with a H_2 Raman shifter, producing several ultraviolet wavelengths.[46] The laser was noisy and slow (10 pulses/s, each 10 ns long), but it worked well enough to explore the UVRR spectra of the nucleic acid bases, singly[47] and in DNA,[48] and the aromatic amino acids, in and out of proteins.[49,50]

Work continued on nucleic acids,[51−53] but proteins and the amide bond became our major UVRR focus. We studied

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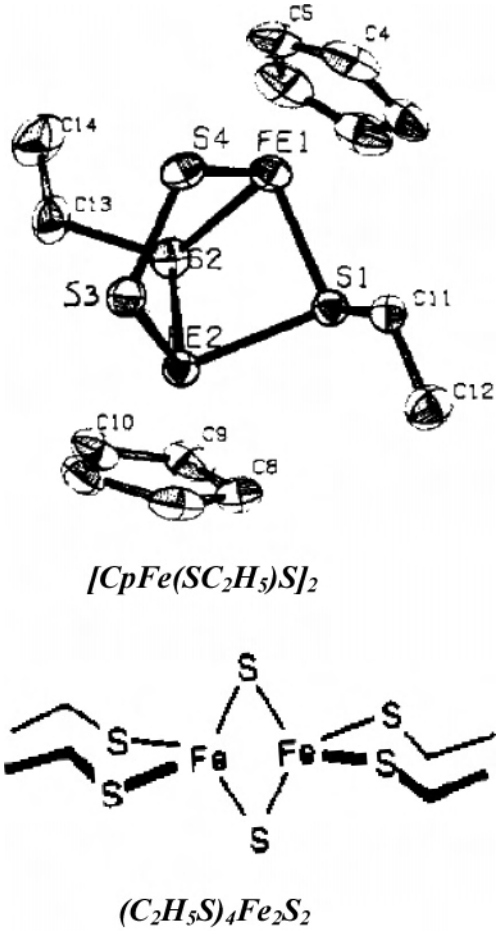

Figure 5. Structure of an early FeS model[30] (top) and of a Holm-type model for [2Fe−2S] proteins[33] (bottom).

the UVRR signatures of tyrosine,[54] of histidine,[55,56] and of proline.[57] Peptide studies were extended deep into the ultraviolet, where amide enhancement patterns and the dependence on conformation could be worked out.[58−60] UVRR was also applied to enzyme mechanisms in a steroid isomerase[61] and in thymidylate synthase.[62]

Porphyrins Proliferate

An Early Surprise: Anomalous Polarization. What really got RR spectroscopy off the ground was the discovery of anomalous polarization by Tom Strekas, which we reported in 1972.[63] Among the complexes proposed for study under my first NIH grant, I had included the recently discovered iridium dioxygen complexes, which were the first reversible nonbiological O_2 adducts. This was to be Tom's thesis, but he soon found that the complexes decomposed in the laser beam before he could get a spectrum. The NIH proposal had indicated that we would study biological O_2 carriers once we had characterized the "simpler" iridium complexes, and in desperation I suggested that Tom give hemoglobin (Hb) a try. This experiment too was a failure; no spectrum was forthcoming.

Just then, Hb expert Chien Ho dropped by the laboratory. When we told him of our frustrations, he asked how we prepared our Hb. Tom pointed to a bottle of powdered Hb from Sigma. Chien shook his head, explained it was probably oxidized, and gave us the procedure for purifying Hb from whole blood. In an act of true scientific dedication, Tom went to our local hospital, had them draw a pint of his own blood, and purified his own Hb. When he put it in the laser beam, he was rewarded with a gorgeous RR spectrum, filled with numerous strong peaks.

We had no idea how to interpret the spectrum, having only a vague concept of what the RR effect was. However, Tom found that the peaks shifted when he deoxygenated the Hb. So, there was clearly chemical information to be deciphered. The big surprise was that some of the peaks had the *wrong* polarization. Every textbook told us that the intensity ratio for scattered light polarized parallel and perpendicular to the incident light was 3:4 (depolarized) or less (polarized). Instead, we had peaks with much higher ratios, with most of the signal appearing in perpendicular polarization; cytochrome *c* showed the same effect (Figure 6). I told Tom he must be mistaken, but he checked the polarizers and stuck to his guns. Moreover, acting on an idea that the anomalously polarized peaks might arise from vibrations having rotational symmetry (A_{2g} in the idealized D_{4h} symmetry of the heme group), Tom worked out the rotational invariants from first principles and showed that their depolarization ratio should indeed be infinite. These modes have antisymmetric Raman tensors and are normally forbidden but become allowed in resonance; they are a specifically RR phenomenon. Our paper on this subject[63] created a stir and brought RR spectroscopy a good deal of attention.

Porphyrin Systematics. The heme RR spectrum is very rich, and many students and postdocs were involved in sorting it out, from systematization of chemical effects with model hemes,[64,65] to our first normal-mode analysis,[66] to assignment of the important iron−histidine stretching mode in Hb.[67] Band frequencies were correlated with porphyrin core size,[68,69] and we used isotopic substitution to unravel the contributions from vinyl substituents[70] and from out-of-plane modes.[71]

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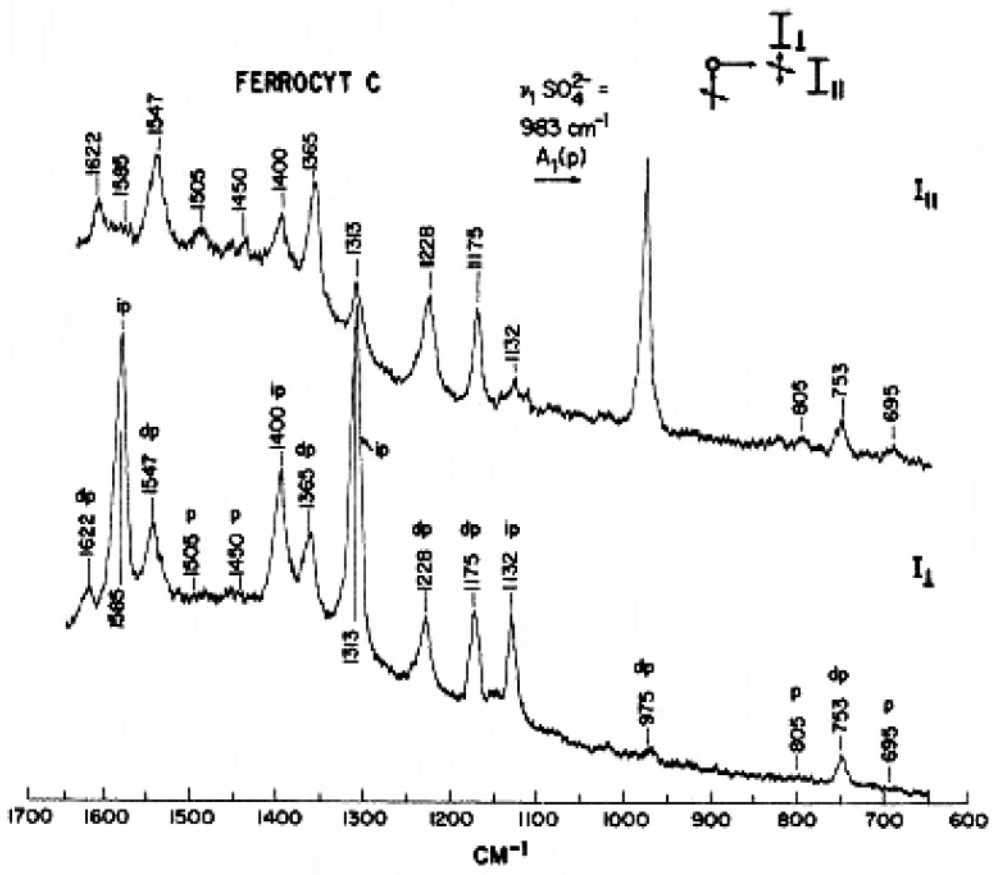

Figure 6. RR spectra of iron(II) cytochrome *c*, with 514.5-nm excitation, analyzed in parallel and perpendicular polarization.[63] Several bands show inverse polarization (ip), whereas the ν_1 band of the SO_4^{2-} internal standard is completely polarized.

Assignment of the numerous porphyrin vibrations was a continuing preoccupation. Jim Kincaid, Roman Czernuszewicz, and Xiao-yuan Li systematically determined isotope shifts for a series of porphyrins, and Xiao-yuan did a heroic normal-mode analysis in order to construct a consistent porphyrin force field that accounted for all of these data, including the out-of-plane modes.[72,73] The force field was later refined further,[74] and theory at the MNDO/3 level was used to investigate the systematics of the porphyrin structure and vibrational frequencies.[75]

Soon after, however, the ab initio density functional theory (DFT) era dawned, and we found that the normal modes of nickel porphine[76] and NiTPP[77] were better reproduced by DFT than by our painfully refined empirical force field. RR intensities were also computed,[78] and we explored the important issue of out-of-plane mode enhancements resulting from porphyrin distortion. These modes became important in our subsequent work on the mechanism of metal insertion via porphyrin distortion in ferrochelatases.[79,80]

Radicals, Excited States, and Energy Conversion. Early on we found Martin Gouterman's four-orbital model of the porphyrin electronic structure to provide a satisfying explanation of the porphyrin resonance enhancement pattern[63] and later of the frequency shifts and Jahn−Teller effects in porphyrin radical cation[81] and anion[82,83] formation. Oxo−metal porphyrins, relevant to peroxidase intermediates, were also characterized, in collaboration with colleague Jay Groves.[84] Later we studied RR spectra of porphyrin and chlorin excited states,[85−87] with an eye toward modeling photosynthetic reactions.[88]

This work dovetailed with a developing project on photoelectrochemical solar energy conversion, using electrochemically polymerized films of vinyl-derivatized ruthenium tris(bipyridine)$^{2+}$ [89] and of protoporphyrin.[90] Photo-

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chemical efficiencies were low for these films, and we decided to test the efficacy of covalently attached donor groups in extending the lifetime of charge-transfer states in cobalt(II) porphyrins, hoping to generate H_2 via the transiently formed cobalt(I). However, picosecond spectral studies on these constructs revealed that the hoped-for charge-transfer states were short-circuited by low-lying d−d states.[91]

Heme Protein Menagerie. We branched out into heme proteins other than Hb. Songzhou Hu carried out complete assignments of the complex RR spectra of cytochrome *c*[92] and of myoglobin[93] via isotopic labeling. Peroxidases were studied intensively[94−96] especially by Giulietta Smulevich, who made them the base of her subsequent research career.

The peroxidases revealed complex patterns of Fe−CO and CO stretching modes in the carbonyl adducts, and we carried out many studies to understand the systematics of these indicator modes,[97,98] demonstrating that the observed variations in many heme proteins and models reflected back-bonding changes associated with electrostatic influences in the heme pocket and not geometric distortion, as had been initially supposed. This led us directly into the longstanding controversy over whether proteins control the CO affinity by sterically hindering the upright binding of CO to the heme. Pawel Kozlowski was able to resolve a conflict between the crystallographers, who found modest bending of the FeCO unit, and the IR spectroscopists, who insisted that the FeCO unit was essentially linear. He showed via DFT calculations that the IR transition dipole was not collinear with the CO bond vector and that the angle allowed by the IR measurements was actually consistent with the crystal structures (Figure 7).[99] Nevertheless, the energy required for modest bending was found to be low, and analysis of kinetic and equilibrium data showed that ligand discrimination by the distal histidine in myoglobin is almost entirely due to a stronger hydrogen bond to O_2 than to CO.[100]

Our attention has recently shifted to heme sensor proteins, in which ligand binding to heme controls the enzyme activity or DNA binding at another site in the protein. In the case of the NO sensor, soluble guanylyl cyclase, we helped established that NO binding induces dissociation of the proximal histidine ligand.[101] We also work out the mechanism and dynamics of CO activation of the transcription factor CooA[102,103] and the mechanism of CO inhibition of the heme-containing metabolic enzyme cystathionine β-synthase.[104]

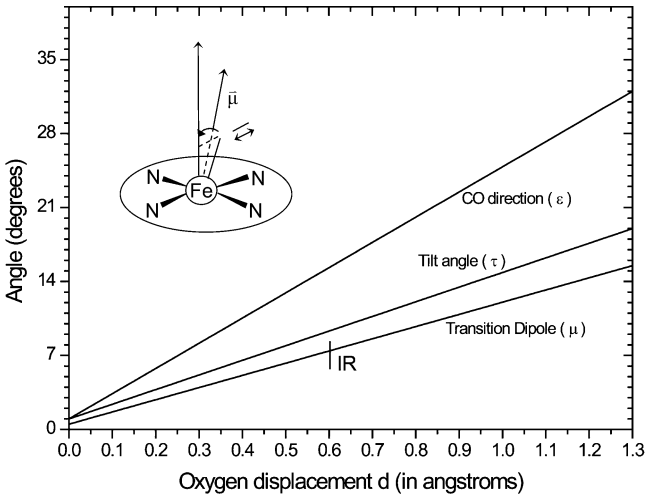

Figure 7. DFT computation of the heme C ↔ O transition dipole direction along a minimum-energy distortion coordinate (bend + tilt), showing the IR-determined angle to be consistent with modest distortion.[101]

A Little Heme RR Lesson

Some background may help the reader appreciate why heme protein RR spectra are endlessly fascinating, and occasionally even useful.

Nature has done spectroscopists a great favor by choosing heme as a cofactor in myriad proteins. The iron−porphyrin complex absorbs visible light intensely, making heme proteins easy to monitor with absorption spectroscopy. In addition, they provide high-quality RR spectra because resonance enhancement scales with chromophore absorptivity. One can record strong RR signals at heme concentrations of 10 μM and below. Lasers are readily available in the 400-nm region, near resonance with the Soret electronic transition that gives rise to the intense absorption. Also, fluorescence, the bane of Raman spectroscopists, is efficiently quenched by the iron ion, whose low-lying d−d transitions rapidly depopulate the excited state. Finally, the heme group has high symmetry, making vibrational assignments straightforward, despite the large number of atoms.

Small wonder, then, that the literature is replete with RR spectra of all sorts of heme proteins.

How Resonance Enhancement Works for Heme. However, what information do these spectra provide?

The answer requires a brief excursion into Raman theory and the heme electronic structure.

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When the exciting light is tuned to an electronic transition, certain Raman peaks are enhanced selectively. They arise from vibrational motions, which shift the chromophore geometry toward that of the excited state.

Near an electronic resonance, the Raman polarizability (proportional to the square root of the intensity) can be expressed as

$$\alpha = A + B \tag{1}$$

where

$$A = \mu_e^2 \sum_v F_v/(\Delta\nu_v + i\Gamma_v) \tag{2}$$

and

$$B = \mu_e \mu'_e \sum_v F'_v/(\Delta\nu_v + i\Gamma_v) \tag{3}$$

In these equations, $\Delta\nu_v$ is the detuning interval, the difference between the laser energy and the energy of a vibrational level, v, with bandwidth Γ_v, in the electronic excited state. Resonance occurs as $\Delta\nu_v$ approaches zero. μ_e is the dipole moment of the electronic transition, and μ'_e is its derivative along the vibrational coordinate, Q, associated with the Raman band being monitored. For strong transitions, μ_e is much larger than μ'_e, and consequently the A term dominates the Raman spectrum. The B term becomes important in resonance with a weak transition, when μ'_e can become larger than μ_e. μ'_e depends on mixing of the resonant transition, e, with an allowed transition, s (called the vibronic or Hertzberg−Teller coupling)

$$\mu'_e = \mu_s \langle s|dH/dQ|e\rangle/(\nu_s - \nu_e) \tag{4}$$

and becomes large when the two electronic transitions are close in energy.

The vibrational selectivity of resonance enhancement is contained in the factors F_v and F'_v, which are products of vibrational wavefunctions (Franck−Condon integrals) between the ground and excited states. They are

$$F_v = \langle 1|v\rangle\langle v|0\rangle \tag{5}$$

where 0 and 1 are the wavefunctions of the zeroth and first vibrational levels of the ground state and v is any vibrational level of the excited state.

$$F'_v = \langle 1|Q|v\rangle\langle v|0\rangle + \langle 1|v\rangle\langle v|Q|0\rangle \tag{6}$$

A nonzero value for F_v requires the excited-state potential surface to shift along the vibrational coordinate, and this is only possible for modes that carry the full symmetry of the molecule (totally symmetric modes). However, F'_v selects for modes that mix the e and s states, and these have symmetries contained in the direct product of the e and s transition symmetries. Thus, a resonance with strong electronic transitions is dominated by totally symmetric modes, but a resonance with weak transitions can bring out nontotally symmetric modes.

The heme chromophore illustrates these principles beautifully. The visible-region electronic transitions are based on π-orbital excitations from close-lying highest occupied molecular orbitals, a_{2u} and a_{1u} (in the idealized D_{4h} symmetry of the porphyrin ring), to a degenerate pair of lowest unoccupied molecular orbitals, e_g* (see Figure 8). These two pairs of excitations give rise to two degenerate electronic transitions, E_u, which undergo configuration interaction. The transition dipoles add up for the higher transition and nearly cancel for the lower one; they cancel exactly if a_{1u} and a_{2u} have the same energy. This is Gouterman's four-orbital model,[105] and it explains the characteristic porphyrin absorption spectrum, with its very strong ~400-nm band, called Soret or B, and its much weaker ~550-nm band, called α or Q. Some of the cancelled intensity (about 10%) can be "stolen" back from the B to the Q transition, via vibronic mixing, producing a ~500-nm vibronic sideband, called β or Q_1.

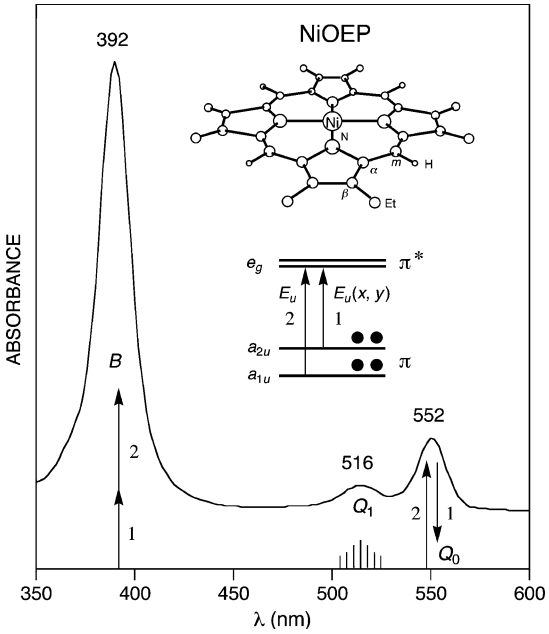

Figure 8. Absorption spectrum of NiOEP (OEP = octaethylporphyrin) and its interpretation via Gouterman's four-orbital model.[105] The $e_g \leftarrow a_{2u}$, a_{1u} orbital excitations (labeled 1 and 2), being of the same symmetry (E_u) and nearly degenerate, interact strongly, with the transition dipoles adding up for the intense B transition at 392 nm and nearly canceling for the weaker Q_0 transition at 552 nm. About 10% of the B band intensity is borrowed by the Q transition, producing the Q_1 vibronic side band of 516 nm, above Q_0 by ≈1300 cm^{-1}, the average frequency of the vibronically effective modes (figure from ref 133).

Gouterman's model also explains the intensity pattern of the heme RR spectra, which vary strongly with the laser wavelength. This variation allows a clear analysis of the porphyrin vibrations, despite the great complexity of the molecular structure. Even if we ignore (for the moment) the axial ligands and the peripheral substituents on the heme (counting only the C atoms attached directly to the ring; Figure 8), the heme group still has 37 atoms and 105 normal modes of vibration (3N-6). However, only nine of these are totally symmetric (A_{1g}). They are the ones that dominate RR spectra when the laser wavelength is near 400 nm, in resonance with the B electronic transition (Figure 9). Their depolarization ratio (intensity ratio for scattered light polarized perpendicular and parallel to the laser polarization) is 1:8.

(105) Gouterman, M. Optical Spectra and Electronic Structure of Porphyrin and Related Rings. In *The Porphyrins*; Dolphin, D., Ed.; Academic: New York, 1979; Vol. 3, Part A, pp 1−156.

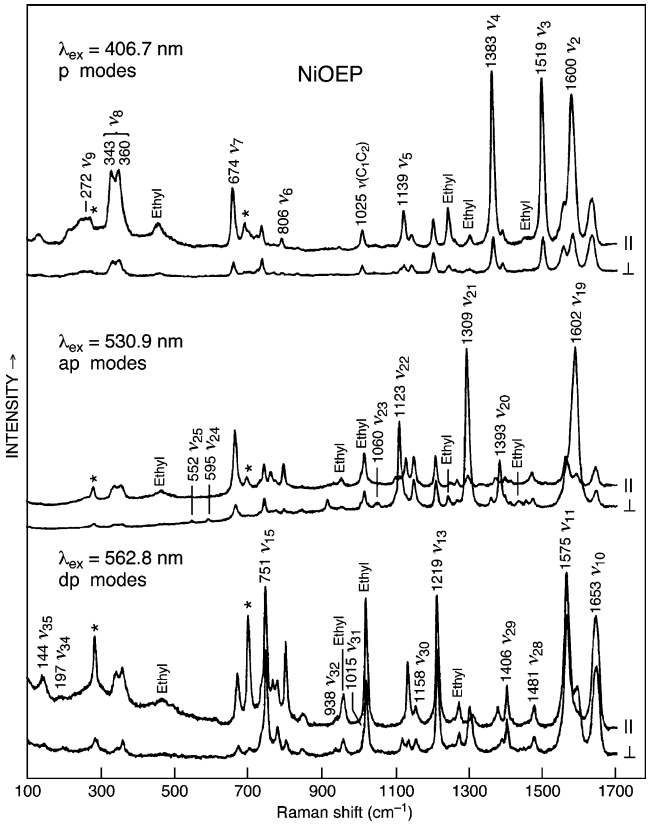

Figure 9. RR spectra in parallel (||) and perpendicular (⊥) scattering for NiOEP,[73] showing the selective enhancement of different modes with different excitation wavelengths. Resonance with the B absorption band (406.7 nm) enhances mainly polarized bands, arising from totally symmetric modes, A_{1g}, although appreciable enhancement is also seen for Jahn−Teller active B_{1g} modes (see ν_{10} and ν_{11}), which are depolarized. Resonance in the Q-band region enhances depolarized (B_{1g} and B_{2g}) and anomalously polarized (A_{2g}) modes and is effective in vibronic mixing of the Q and B electronic transitions. Due to interference effects, the A_{2g} modes are brought out most strongly via excitation between Q_0 and Q_1 (530.9 nm), while the B_{1g} and B_{2g} modes are brought out more strongly with excitation outside the Q bands (568.2 nm). The mode labels ν_i refer to porphyrin skeletal modes;[134] modes assignable to the ethyl substituents are also seen, as indicated (figure from ref 133).

When the laser is tuned to the Q bands, entirely different modes become dominant (Figure 9). They are the vibronically active modes, the same ones that produce the Q_1 absorption intensity. Because the B and Q transitions both have E_u symmetry, the symmetries of the vibronic modes are given by

$$E_u E_u = A_{1g} + A_{2g} + B_{1g} + B_{2g} \qquad (7)$$

However, the A_{1g} vibrations are ineffective in vibronic mixing because of the high molecular symmetry, and the remaining modes dominate the Q-resonant spectra. Depolarization ratios are 3:4 for B_{1g} and B_{2g} bands but infinite (*inverse* polarization) for A_{2g} bands. Inverse polarization is the unexpected effect discovered by Strekas in our first study of hemoglobin and cytochrome *c* (although it had been predicted in a footnote by Placzek in 1934!).[63] Textbooks had asserted that nontotally symmetric modes all have [3:4] depolarization ratios, but this is clearly not true for A_{2g} modes. The reason is that they have rotational symmetry (R_z) and *anti*symmetric polarizability tensors ($xy = -yx$). They literally rotate the plane of polarization, and all of the intensity is found in perpendicular scattering. (In actual spectra, some parallel intensity is observed because the symmetry is not fully D_{4h}. Depolarization ratios are nevertheless >3:4, and the bands are called *anomalously* polarized.)

A_{2g} mode activity is purely a resonance phenomenon. These bands disappear off-resonance. The reason is that the individual terms in the summation of eq 3 change signs as the laser sweeps through $\Delta\nu_v = 0$, and successive terms can add or subtract. Because of tensor antisymmetry, adjacent terms add for A_{2g} modes when the laser is tuned between them but subtract when the laser is tuned away from them. Thus, the intensity cancels off-resonance. The opposite happens for B_{1g} and B_{2g} modes, whose tensors are symmetric ($xy = yx$). Their intensity is minimized between resonances but survives off-resonance. These opposite tendencies permit tuning of the laser to selectively enhance A_{2g} or $B_{1g/2g}$ modes, as is illustrated in Figure 9.

Because of these symmetry effects, the RR modes of the heme group have been thoroughly catalogued.[72,74] Symmetry greatly simplifies the spectra and allows reliable mode identification. It also hides information, however. Many of the 105 modes do not appear in the RR spectra at all. A total of 54 of them are "u" modes and are IR but not Raman active. They have been assigned from IR spectra of metalloporphyrins, but IR spectroscopy is severely limited for heme proteins by the strong IR absorption of water and of interfering protein modes. There are also out-of-plane modes (some "u" and some "g"), which are not resonance-enhanced, because they cannot couple to the resonant electronic transitions, which are polarized in the heme plane. However, some "u" modes, both in-plane and out-of-plane, do appear in heme RR spectra because of symmetry-lowering effects.[71−73,106] These can arise from the intrinsic asymmetry of the porphyrin substituents, asymmetry in the axial ligands, out-of-plane distortions of the porphyrin ring, and electrostatic asymmetry of surrounding residues in proteins.

Porphyrin RR Bands Report Iron Stereoelectronics. Although the heme RR bands arise from vibrations of the porphyrin ring, they nevertheless are sensitive to ligation changes at the central iron. Early on, it was recognized that frequency shifts in several modes were linked to changes in the oxidation and spin state of the iron.[64] The main mechanism for this chemical sensitivity turned out to be stretching of the porphyrin bonds to accommodate changes in the iron size and its displacement from the center of the ring.[68,69] The porphyrin ligand-field strength is poised so that the Fe^{II} or Fe^{III} spin state is tuned by the axial ligands. In heme proteins, one of the axial ligands is usually imidazole (histidine side chain), although thiolate (cysteine), thioether (methionine), and amine (lysine or the protein N terminus) are also encountered. If the sixth ligand is strong-field (cyanide, thioether, or, for Fe^{II}, CO, NO, and O_2), the iron is low-spin and the d electrons (5 and 6 for Fe^{III} and Fe^{II}) are all in π-bonding orbitals (t_{2g}). However, if the sixth ligand is weak (H_2O or F^-) or absent, the iron is high-spin, and one electron occupies each of the σ-antibonding orbitals, lengthening the bonds and expanding the porphyrin ring. If the sixth ligand is absent, the iron is displaced toward the fifth ligand, partly relaxing the ring expansion.

The ring-size dependence of several porphyrin bands has been calibrated with metallopophyrins of known structure,[68,69]

(106) Li, X. Y.; Czernuszewicz, R. S.; Kincaid, J. R.; Spiro, T. G. *J. Am. Chem. Soc.* **1989**, *111*, 7012−7023.

and the data for heme proteins fall quite predictably on these correlations. Thus, the positions of these "spin marker" bands are routinely used to monitor the ligation status of the heme group.

The strongest band in Soret-resonant RR spectra, designated as ν_4, was early recognized as a marker of the oxidation state, having characteristic narrow ranges for Fe^{III} and Fe^{II}, regardless of spin state.[64] The reasons for this behavior are complex, involving counterbalancing influences of the ring size and of π bonding. For CO, NO, and O_2 adducts of Fe^{II}, the ν_4 position is close to that of Fe^{III} because substantial charge is transferred to these π-acid ligands via back-bonding. Because ν_4 is intense, it serves as a useful monitor of ligation for these adducts.

Axial Ligands Report Protein Influences. Because the axial ligand bonds are perpendicular to the heme ring, their vibrations are not normally detectable in RR spectra because the resonant electronic transitions are polarized in the plane of the ring. However, there are important exceptions.

Because of back-bonding, the vibrations of bound XO (X = C, N, O) are resonance-enhanced. The π^* orbitals on XO compete with those on the porphyrin ring (e_g^*) for back-donation of the Fe d_π electrons. Excitation of the porphyrin $\pi-\pi^*$ transitions weakens the porphyrin but strengthens XO back-bonding. Consequently, there is a shift of the excited-state potential along Fe−X and X−O stretching coordinates.

The RR bands associated with Fe−X and X−O stretching have proven to be valuable probes of protein interactions with the heme, especially for CO adducts.[107] The extent of back-donation is sensitive to the electrostatic influence of polar residues in the heme pocket. Positive polarity increases back-bonding, while negative polarity decreases it, with anticorrelated changes in the positions of ν_{FeX} and ν_{XO}; increased back-bonding strengthens the Fe−X bonds and weakens the X−O bonds. A series of myoglobin variants with distal residue replacements have provided a standard back-bonding correlation for CO adducts,[107] which has been much used in assessing distal influences in other proteins.

NO adducts have seemed not to follow this pattern,[108−110] but recent computational modeling has revealed that the complexity apparent in the NO data results from the ambidextrous nature of hydrogen bonding to the bound NO.[111] Because $Fe^{II}NO$ is bent, with a partially filled nonbonding (sp^2) orbital on N, hydrogen bonds can be stably formed with either N or O, despite the greater charge on O. H···O bonds produce increased back-bonding, with anticorrelation of Fe−N and N−O bond strengths, but H···N bonds weaken both Fe−N and N−O bonds by drawing more electron density into the sp^2 orbital. Which end forms the hydrogen bond depends on the geometry of the hydrogen-bond donor in the heme pocket, an effect that is apparent in the vibrational data.

Another important RR band is that associated with the bond to the proximal ligand, the only bonding connection between the heme and the protein.[67] Usually the associated vibration is not resonance-enhanced, but five-coordinate iron(II) heme is an exception, and iron−histidine RR bands have been reported for many heme proteins in this state. The displacement of Fe^{II} toward the heme plane when this bond is stretched evidently shifts the excited-state potential enough to produce appreciable enhancement, The position of this vibration reflects the strength of the iron−histidine bond, which is variable among heme proteins, and is an important determinant of the heme chemistry.

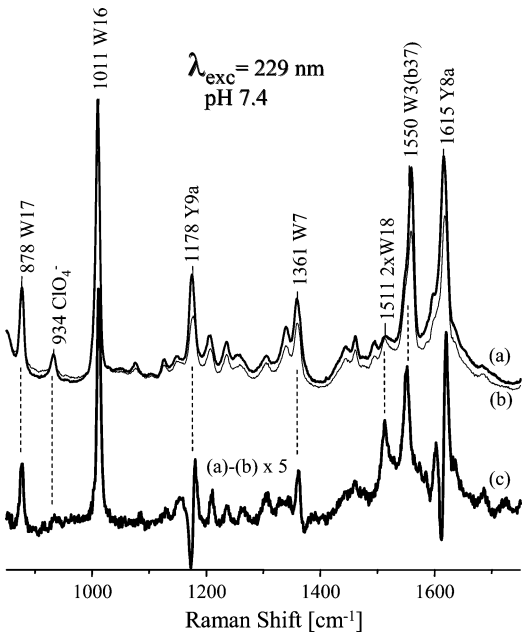

Figure 10. UVRR spectra of Hb in (a) deoxy (T) and (b) CO (R) forms. The difference spectrum (c) shows tyrosine (Y) and tryptophan (W) signals, which are associated with hydrogen-bond changes between the T and R quaternary structures.[117]

There are many more strands in the heme protein RR fabric, which can be found in the cited literature. For now, we return to the story of the Princeton Raman program.

The Time Dimension

When Woody Woodruff joined the group, he had a burning ambition, which he has pursued throughout his research career, namely to add a time dimension to vibrational spectroscopy. He realized that harnessing the structural specificity of RR spectroscopy to kinetic studies would form a powerful combination.

At the time, we had no pulsed lasers to provide time resolution. So, Woody constructed a flow system with a dual syringe pump and aimed the Raman laser downstream of the mixing chamber to produce a spectrum of horseradish peroxidase compound II, the second intermediate of the peroxide reaction.[112] Soon we used Jim Nestor's pulsed-dye laser CARS apparatus to show that that the iron atom was displaced from the heme plane within 6 ns after photolysis of the CO adduct of Hb.[113] Later we pressed the time resolution of this experiment into the picosecond regime using a rapid-flow technique[114] and, subsequently, the HbCO

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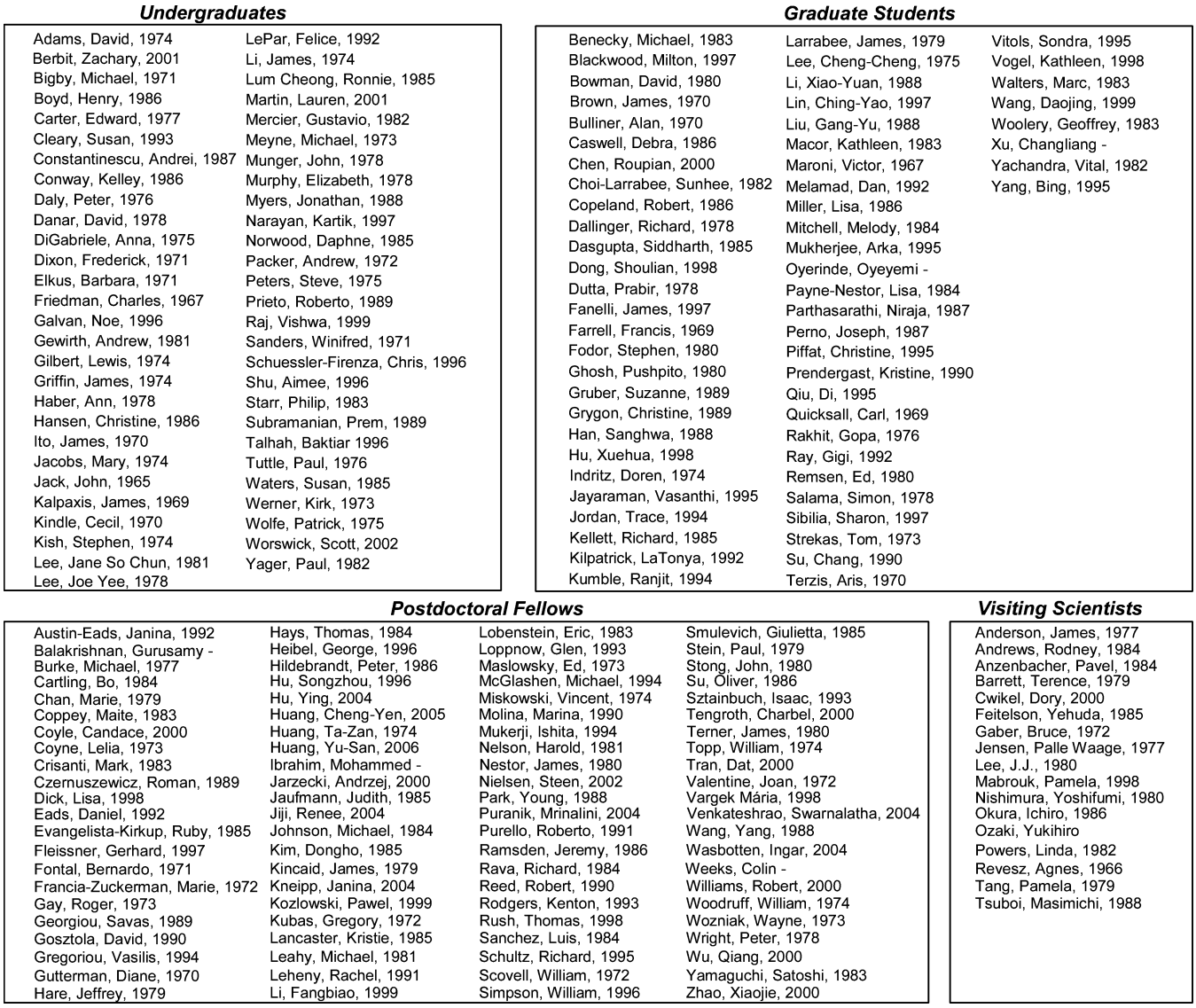

Figure 11. Colleagues of Thomas G. Spiro.

photoproduct was characterized more fully with the 7-ns Nd:YAG laser that we acquired for UVRR studies.[115]

Truly time-resolved RR spectroscopy on the nanosecond time scale became possible with the acquisition of a second pulsed laser, which could be delayed electronically. We realized that ultraviolet excitation would enable us to study protein dynamics by spatial reference points, in the form of the aromatic residues. Inititial attempts to do this on Hb led us astray;[116] the signal-to-noise ratio of our 10-Hz Nd:YAG lasers was just not up to the task.[117] The situation improved dramatically when we traded up to 300-Hz XeCl excimer-dye lasers. Kent Rodgers implemented this system and introduced key sampling improvements.[118] The resulting difference spectra were sufficiently resolved (Figure 10) to allow structural interpretation, and subsequent studies produced the first structural model for the allosteric pathway connecting the R and T states.[119−121] Complementary studies were carried out with a new time-resolved Fourier transform IR spectrometer.[122,123]

The excimer lasers helped us to produce important results, but they were difficult to operate and broke down frequently. We switched to a solid-state system, with Nd:YLF-pumped Ti:sapphire lasers, whose frequency could be shifted into the blue and ultraviolet regions via harmonic generation in mixing crystals. The lasers are stable and operate at 1-kHz repetition rates, producing spectra of superior quality.[124] We were able to sharpen the structural interpretation of the Hb time course and found an important new intermediate on the pathway.[125] We examined site-directed mutants designed to test our pathway model and came up with exciting new

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results, implicating dynamic cooperativity at the level of subunit dimers within the tetrameric structure.[126,127] Our old friend Hb continues to surprise and delight us.

Our new laser technology provides access to the deep ultraviolet, where amide bands of the protein backbone are optimally enhanced.[128] Gurusamy Balakrishnan proposed that we look at protein folding via laser-induced temperature jumps. The *T*-jump technique allows access to the early events of protein folding and unfolding and had been applied with fluorescence and IR monitoring of the relaxations, but UVRR spectroscopy promised multiple structural probes via the several amide and aromatic residue bands. Bala negotiated the design of a novel Nd:YLF-pumped OPO laser, operating at 1 kHz, to match our ultraviolet probe laser.[129] The system worked beautifully, and we have been applying it to a series of peptide and protein-folding issues.[130−132] The technique is particularly promising as a way to characterize local unfolding events that may play an important physiological role in protein function.

Coda

This brief account has braided the main strands of the research program that I have been privileged to pursue. I have pushed and pulled stories of our many projects into a semblance of a narrative. Research, like life, is seldom straightforward, and my attempt to provide some coherence to the story required leaving out many side strands, some of them quite interesting in their own right, projects launched and, for a variety of reasons, not pursued very far. Likewise, although I have named a few of the players in our research drama, the narrative leaves out many individuals who contributed importantly to the program over the years; some of their work is cited in the references.

A research group has a life of its own, quite independent of the schemes and wishes of its director. I have been blessed with a wonderful collection of students, undergraduate, graduate, and postdoctoral, and of visitors. They have been the lifeblood of the program and have pitched in together, era after era, to make the laboratory work and keep morale high. I have watched with pleasure as they pursue independent paths in science and in life. They are my extended family.

I list their names in Figure 11 in thanks and appreciation.

I also thank my Princeton University faculty colleagues, and a wide circle of collaborators, for their friendship and support over the years.

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