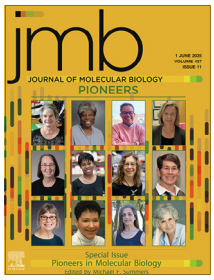

From Rat Tails to Glycoproteostasis: Motivated by Biology, Enabled by Biophysics, and Lucky ☆

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Abstract

In this article I tell the story of my career path and how I have come to focus my research on protein folding in the cell. My early fascination with protein folding began during my undergraduate research. My graduate work exploited reductionist approaches to explore structural features in proteins by using cyclic peptide models of β-turns. My career trajectory from these early days to present, described in the first section of this article, illustrates the importance of pursuing the scientific questions that one finds most exciting and seizing professional opportunities that enable these questions to be tackled productively. In addition, this trajectory shows how serendipity can shape a career path. The second section describes the extraordinary scientific discoveries I have witnessed in protein folding during my career. Here I explain how I was drawn into the world of protein folding in the cell. This turning point allowed me to participate in the explosion of research on molecular chaperones in the early 90′s and to help elucidate the nature of chaperone-substrate recognition, a problem I continue to focus on. Examples of our research contributions are presented in the third section, with a perspective on major challenges for the future offered in the last section. Throughout my career I have engaged in many collaborations; each has opened new scientific doors. Importantly, I seek to instill in my trainees the same excitement about research that I feel and to foster their growth as scientists and their discovery of their own passions and talents.

My Career Path, Shaped by Opportunities Seized

When I was invited to contribute an article to this series in JMB, my first reaction was “pioneer”?? how so? My second reaction was hesitation, as I’m not very comfortable talking (writing) about myself. . .it is much easier to talk (write) about our most recent and most exciting results! But a bit of introspection never hurts, and if someone can find my career journey informative or helpful, all the better. So here goes: My father was a civil engineer, and he attempted to share with his children his passion for design of bridges and power plants. This led to family trips punctuated by visits to particularly exciting (?) bridges and many tours through the power plants whose construction he had overseen. Also, the expectation was that my older brother (Peter, 8 years older) and sister (Molly, 5 years older) and I would be involved in all aspects of building things more locally, as in our yard (pouring concrete, building a shed, constructing trusses for our new house). As the youngest, I aspired to keep up with

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my siblings and thus was born my proclivity for over-achievement... even when ill-advised. But especially my relationship with my older brother (an eminent astronomer who worked on planetary atmospheres in his career at Cornell) fed my curiosity and awareness of science broadly defined. We spent many hours building radios and telescopes, watching celestial events (favorites being meteor showers and Jupiter's moons) and discussing what it means to be "alive". I also credit my mother with the absolutely unquestioned expectation that I would have a career and be treated the same as my big brother in terms of the path I might follow. [It was family lore that my father asked her to define a parabola on their first date, and her ease with doing this led to his continued pursuit of her as a life partner].

In addition to family, my high school teachers were a source of great inspiration to me. They accomplished the all-important dual goal of fueling my interests and letting me know I had ability to pursue them. I thank them, as I was encouraged to follow my passions in science and math. I recall one high school activity that was particularly influential: My high school physics teacher arranged to have a group of us go to a grade school in the nearby city of Holyoke and teach sixth graders on the topic of water and surface tension, showing the students how the experimental method could shed light on the fundamental properties of something they lived with every day. It was great, because the necessary supplies were simply water, soap and wash bottles. The students were to observe when a stream of water emerging from the wash bottle broke into drops. Then soap would be added and the experiment repeated. Reducing surface tension obviously led to a delay in when the water stream broke into drops, if at all. This was cool science and gave a lot of room to allow us high school students to "teach". I loved it. The downside was that giving wash bottles to a sixth-grade class also led to very wet chaos. But for me, I knew from this experience and others that I absolutely love teaching.

My mother went to Mount Holyoke, as did our closest neighbor in Wayland, MA (where I lived from age 3 to 11), Mrs. Benjamin. So I planned from a young age that I would do the same. It was a fortuitous but not carefully considered choice on my part. I loved my time at Mount Holyoke where we were given a "sense of possibility". We could do anything we chose to. I majored in chemistry and took nearly all the biology classes offered. And I did undergraduate research on the folding of rat tail collagen. The tails were "harvested" from expired rats that had been used in psychology experiments. I had a lab of my own about the same size as most junior faculty. When a new electron microscope was installed on campus, I was one of the first major users, spraying my collagen samples onto grids and shadowing them for imaging. It was a playground for me. In the summers after freshman and junior years, I worked at Harvard Bio Labs with Alwin Pappenheimer, an immunologist and a wonderful person who taught me many things but mostly encouraged my growth as a scientist. I connected with Papp, as he was known, after I had knocked on every door in the Harvard Bio Labs, seeking a summer research position. His door was literally the last. He hired me because he had dated a Mount Holyoke woman when he was an undergrad at Harvard and my inquiry led him to flash back to driving his Model T out to South Hadley, repairing several flats along the way. In the summer after my junior year while working at Harvard, I wandered down Mass Ave by MIT and saw a poster for the International Congress on Pure and Applied Biophysics (this would be 1969) with speakers including Paul Flory, V. S. Ramachandran, Harold Scheraga, Aaron Katchalsky, and many more... I had had some exposure to biophysics through a course at Mount Holyoke and at that moment the die was cast. I attended the Congress and was completely won over. That was my love: the intersection of physics, chemistry and biology. Thus, I applied to graduate schools in biophysics and joined the program at Harvard, overseen by the Committee on Higher Degrees in Biophysics.

[You must have noted from the story thus far the major role serendipity has played in my career path. If you haven't noticed it yet, the next anecdote should do the trick.] The centroid of the Biophysics program was at Harvard Med, where the director AK Solomon had his laboratory. But many classes were held in Cambridge. Thus, in my first semester I frequented the yellow school bus that ran between the Med School and Cambridge campuses. I was feeling a bit lost about how to find an adviser for my Ph.D. work, when I heard the word "collagen" from the back of the bus. I rushed back and found the source: Barbara Brodsky, then a more senior student in Biophysics, was discussing her work. I learned that she was working with Elkan Blout, and I jumped all over the possibility of working in his lab as it seemed to fit my interest in molecular biophysics. This turned out to be a wonderful choice: Blout was a supportive, kind, warm and highly globally connected researcher with a keen interest in using chemistry to ask questions about how amino acid sequence governed protein conformational preferences. Moreover, this was the era in his lab when modeling conformations using cyclic peptides and exploiting the newly developed power of high field nuclear magnetic resonance (NMR) were the main focuses. I made many cyclic peptides by solution phase synthesis, harnessed every spectroscopic and computational tool I could find, and received guidance from top-

notch postdocs who were in much larger number in the lab than grad students. It was productive and I encountered no bumps in the road at all as a woman in science, for which I thank Elkan Blout and the whole Blout lab.

But in my somewhat impatient style, I thought I was ready to leave my graduate position early in my fourth year and started looking for a postdoc. But my eyes were drawn to faculty positions as well, particularly when I saw that Amherst College was hiring. This, I thought, was my dream, as Amherst is a superb primarily undergraduate school, down the road from Mount Holyoke. I was ecstatic when hired! Little did I know that I was making a number of poor decisions, but somehow I survived. First problem, when the time came to join the faculty at Amherst, I hadn't actually completed writing and defending my thesis, making the first semester of my faculty appointment very challenging (please note that I was also only 26 years old). This is a time when many challenges are presented to a new faculty member, and I also had to drive back and forth to Boston, finish writing, defend... Also, Amherst, which had been an all-male institution, was joining other elite all-male colleges and universities in deciding to become co-educational; the decision was made during my first semester at Amherst. I was the only woman faculty member in the sciences and math, and in fact had few female colleagues (only one, Rose Olver, tenured). It was not the easiest time (see https://www.amherst.edu/academiclife/colloquia/colloquium-seminar-archives/women_teaching/early_bios/node/330595), particularly as I had not previously experienced an environment where I had to prove myself. In addition, I became very involved in the campus transition to co-ed, coaching the women's cross-country team, serving on campus safety committees and trying to raise the consciousness of the campus to the major change that was happening, *etc*. Nonetheless, my own career was successfully launched: I was awarded an R01 NIH grant and received support through the NSF Research Instrumentation program for a state-of-the-art NMR instrument. And so began my independent research program focusing on what I knew and loved at that point: cyclic peptides (see #1 below). But the fact that I did not do a postdoc (because I thought I knew what I wanted and had found it) deprived me of research breadth and the happy period when you gain insight from a new mentor, who also pays the bills. By good fortune, Amherst College had a very nice sabbatical policy, and I could do a sabbatical leave after 3 years of service. I had met Jean-Marie Lehn (1987 Nobel Laureate for supramolecular chemistry) on a couple of occasions. I also had a love of everything French (still do), including mastering the language. Thus, I spent a year carrying out research in Labo Lehn in Strasbourg, France, and being inspired in new research directions by Jean-Marie Lehn. My love of Strasbourg and the many friends I made then have stayed with me since. I also do my best to maintain my French speaking ability. Most importantly, stepping away from Amherst cleared my mind and helped me realize that I wanted to be at a research intensive (R1) institution with graduate trainees in my lab.

Serendipity intervened again: I was following ads for faculty positions and saw that the University of Delaware sought a biophysical chemist. Wow. Me. I applied, was interviewed, and was hired... not realizing that they had posted the ad because they were required to do so, as they had intended to move a senior postdoc into a faculty position. But I came along. All's well that ends well: that person obtained a good position elsewhere! And for me, I had eight wonderful years in which my love of teaching was fulfilled along with nice development of my research program. My next door office neighbor was Joe Noggle, who co-wrote the book on the nuclear Overhauser effect (J.H. Noggle and R. E. Schirmer, "The Nuclear Overhauser Effect: Chemical Applications:"). With solid-state NMR spectroscopist Cecil Dybowski (next office down) we founded the Blue Hen NMR Symposium, which ran long past the eight years when I was at Delaware and was known for its great speakers as well as an excellent and lively wine tasting.

More serendipity: My major unmet need at Delaware was a high field NMR (at that time, >250 MHz). Al Gilman (then Chair of Pharmacology at UT Southwestern, and to receive the Nobel Prize in Physiology or Medicine, 1994, for G-protein signaling) was sitting on NIGMS Council and read one of my tear-stained letters trying to get funding for a 500 MHz NMR when I was basically to be the only major user. He hypothesized, correctly as it turned out, that he could attract me to UT Southwestern by giving me a 500 MHz NMR. While moving to Dallas was a bit of an unwelcome prospect for this New Englander, being recruited by an amazing array of scientists (including Al, 1985 Nobel Prize winners in Physiology or Medicine, Mike Brown and Joe Goldstein, and a host of other fantastic researchers) was so persuasive that I realized I simply had to accept for the sake of my science. The scientific opportunity was indeed unbelievable. And the six years I spent at UT Southwestern proved pivotal to my research trajectory. They coincided with the discovery of molecular chaperones and the explosive research wave that ensued. I was able to catch the wave (Figure 1)! My work was buoyed by collaborations with the high quality UT Southwestern faculty as well by the Howard Hughes Medical Institute structural biology faculty recruited to UT Southwestern at the same time I was there, with hires of Hans Deisenhofer (Nobel Prize in Chemistry, 1988), Steve Sprang, Betsy Goldsmith,

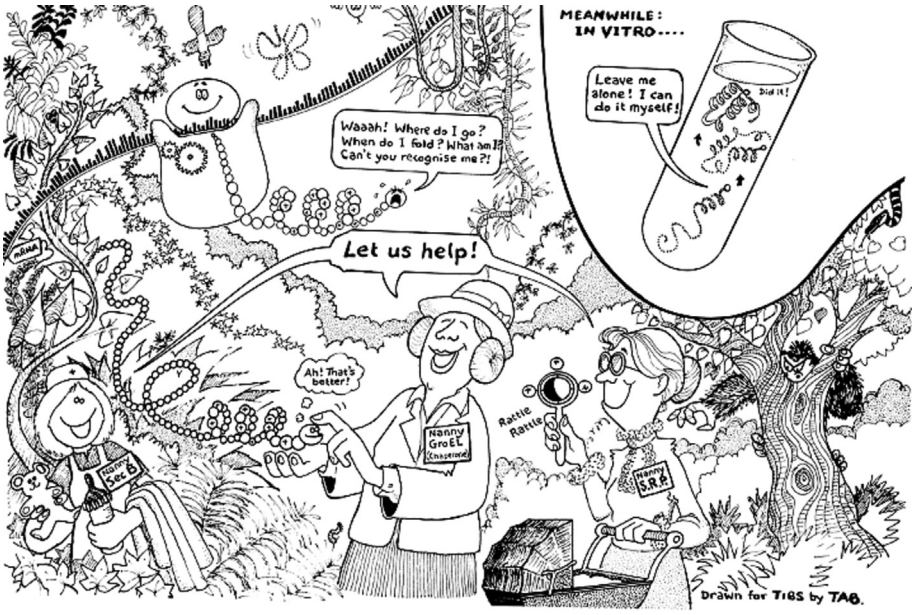

Figure 1. A bit of history in the early days of chaperones: Cartoon figure created by the TiBS artist to accompany a review submitted in 1991. We wished to indicate that nascent polypeptides are assisted in their folding and targeting by molecular chaperones, which were likened to nannies by the artist! Figure from.[52]

and others. Together with other terrific biophysical faculty, we founded the now thriving Graduate Program in Molecular Biophysics, which in fact has become a department! It was a great time in many ways. But after some time, I felt an itch that I needed to scratch to get back to snow and my roots, and to be back on a campus with undergrads.

When the University of Massachusetts Amherst posted an ad for someone working in NMR, I took the bait and called Pete Lillya, chair of the search and a friend from way back. I was interviewed, but my proclivity for organizing things and leading new initiatives seem to catch the eye of the Chemistry Department Head search committee, who convinced me to consider the position of Department Head. The then Dean Linda Slakey sweetened the pot by promising ten new faculty hires and a major renovation of department space. I succumbed.

I have greatly enjoyed my time at UMass Amherst. The Dean's promise of new hires was fulfilled, and I was able to influence faculty hiring to enhance research strengths on campus and make it an even more stimulating environment to work in than it already was. After I was Head of Chemistry for five years, Dean Slakey asked me to be Head of Biochemistry & Molecular Biology, with another bolus of hires. In a way, from my perspective, through these two leadership positions I was able to help enrich the campus with talented colleagues I could enjoy working with and also to foster the establishment of an institutional research focus in protein homeostasis, writ broadly. We became known for this, which catalyzed hiring of many talented faculty. A new Institute for Applied Life Sciences (IALS) was established and received generous funding from the state to upgrade facilities. Peter Reinhart, formerly of Proteostasis Therapeutics Inc. was hired as Director of IALS. With the IALS state investment, we set up a biophysical lab that houses every instrument we could imagine using to measure binding and stability, we upgraded the NMRs, and we established a powerful imaging center. Fortuitously, the long-standing logjam in new building on campus was broken, and several of us were able to be involved in the design of two wonderful new interdisciplinary teaching and research buildings, the Integrated Sciences Building and the Life Sciences Laboratories. Here for the last 31 years I have been happily conducting research, working with excellent colleagues, and facilitating the training of many talented undergraduates, graduate students, and postdoctoral fellows.

Throughout my career I have maintained a devotion to my outside activities: primary among them is horseback riding. I once competed in eventing, which requires jumping over cross-country courses as well as performing precise dressage tests. I have in the last decades focused on dressage, and I continue to. When I have had the means, I have owned horses. In early days as an assistant professor, I rode others' horses. I am an active person beyond the riding: biking, birding, hiking, etc., but riding is primary. In a happy twist

of fate, while I was at UT Soutwestern I met a guy (John Pylant) who rides seriously, and dressage to boot! He also has a good sense of humor (essential to hang around me), enjoys a highly active lifestyle, and is immensely supportive of the crazy life I have. We hitched our lives together and now are striving to age together, gracefully if possible.

Lessons from this career path? Seize opportunities whether for new scientific directions, new positions or new collaborations; recognize in yourself what matters most to you, which for me started with a love of teaching and has led to unending joy from working with trainees; be open to new things and dare to dive into them; accept circumstances that seem as though you are being favored with good luck to be choices that you can allow yourself to make in order to expand your horizons; hang out with smart people who keep you on your toes. Most of all: have fun doing what you have chosen as your career! And keep your life balanced so that you are healthy and sane!

A Serendipitous Scientific Journey, or How the Protein Folding Field Has Evolved on My Watch

When I started my Ph.D. research in 1970, global leaders in protein structure and folding were inspired by the first few atomic level structures of proteins and consequently focused on defining the sequence preferences of the most common secondary structures and spectroscopic tools to define and characterize them. This is what inspired my work in the Blout lab on cyclic peptides as models for turns. What has happened in the intervening half century is nothing short of astonishing! For one thing, the number of atomic-level protein structures that have been solved and are now accessible to all in the PDB has risen to more than 120,000. X-ray crystallography has become a routine method with which to solve many protein and nucleic acid structures, and cryo-electron microscopy (cryo-EM) and structure prediction have opened the door to many, many more structures of increasing complexity.

In the area of protein folding, Christian Anfinsen's seminal work[1] was published during my formative years and established the crucial concept that the amino acid sequence of a protein is sufficient to direct it to adopt its native fold. Protein folders were inspired to elucidate the mechanisms underlying the folding process, but in the early days were quite limited in their targets and focused on readily prepared proteins, many of which could be bought from Sigma! The work that was done in many labs across the globe studying protein folding was pivotal to our current paradigms, and there is no question that current thinking about folding was shaped in fundamental ways. To wit: from kinetic and thermodynamic descriptions of folding pathways to landscapes and funnels. However, I recall vividly a conversation with a group at a Protein Society dinner many decades ago when I raised the question "how does folding occur in the cell"? I was silenced quickly by the statement that it works *in vitro*, and the native structure can be attained, so the *in vivo* mechanism is not of interest. What was bothering me was the fact that most of the proteins being studied were secreted proteins, and thus they were synthesized with signal sequences that targeted them to the secretory pathway, and they then traversed multiple cellular compartments *en route* to their final destination. I was convinced it was important to consider what happened in the cell.

Fueling my focus on in-cell protein folding at that time was a chance encounter with Tom Silhavy (now of Princeton, then at NCI) when he came to give a seminar at the University of Delaware. He presented a wonderful story about his genetic studies of bacterial signal sequences and the components that guide proteins from the gram-negative bacterial cytoplasm to the periplasm or outer membrane. With his then student Scott Emr (now at Cornell), he found a defective mutant version of the LamB (maltoporin) signal sequence that could be restored to function by second mutations, and they proposed this was because function in this sequence required that it adopt an α-helix.[2] Based on my background with peptide synthesis, I boldly said that their hypothesis was testable using synthetic signal peptides. Thus was born a really fun collaboration between the Silhavy lab and mine and a several decade-long research focus in our lab on the properties that conferred the ability of sequences to target proteins for secretion. It also catalyzed my fixation with *in-vivo* protein folding. I have to say that I was a bit too brash in telling Tom Silhavy we could make peptides to test his hypothesis about secondary requirements on signal sequences, as these were at the time long-ish peptides to make (*ca*. 25 amino acids) and most amenable to solid-phase peptide synthesis, which my lab had not done. I sent then graduate student Martha Briggs off to Bruce Merrifield's lab at Rockefeller University to learn enough to bring home the capability for us to do solid-phase peptide synthesis. Which she did. And from then on, we were enabled (including buying early semi-automated synthesizers, after realizing the wrist-action shaker used in Merrifield's lab was not very high throughput!). Most importantly, this launched a two-decade research project in the lab on signal sequences and how their properties enable them to target proteins for export from bacteria or entry into the ER in mammalian cells (see #2 below).

When I think about the most impactful times in my career, I am drawn to a year-long sabbatical that I was able to do towards the end of my time at Delaware with support from the Guggenheim Foundation. I split the year in three: a four-month stint in Tom Silhavy's lab, a four-month stint with

Peter Walter at UCSF relating my bacterial signal sequence work to the mammalian secretory world, and a four-month stint at Smith-Kline (the name at that time!) where Stan Opella was leading a group focused on applying NMR methods to pharmaceutical targets. NMR had just experienced a revolution based on the development of multi-dimensional methods in Richard Ernst's lab.[3] and the application of these to protein structure in Kurt Wuthrich's lab. I knew I needed to harness these methods, and Stan had recruited Art Pardi and Luciano Mueller to join him. I was so fortunate to be able to be "tutored" by this group in then state-of-the-art NMR methods.

In both the Silhavy and Walter labs, my biophysical perspective was broadened tremendously. From this fabulous year onward, our lab made sure to motivate our work by the biological question it addressed... and we appreciated so much more the experimental approaches that were needed to answer questions in physiologically meaningful ways as well as the logic of living systems. So different from cyclic peptide conformational studies I did as a graduate student and young faculty member! But our biophysical thinking was welcomed in both these labs... true synergy.

The consequence of this amazing sabbatical was a preparedness to benefit from the environment at UT Southwestern and the exciting discoveries in protein folding that probably couldn't have been achieved any other way. Thus, I was intellectually ready to see the impact of the earliest work on molecular chaperones emerging from Art Horwich's, Ulrich Hartl's, John Ellis', and George Lorimer's labs. This positioned us to apply our biophysical methods to answer some fundamental question about chaperone structure/function. There are epochs in every lab, and the early chaperone epoch in mine, spearheaded by Sam Landry (now at Tulane Medical School), led to significant new insights in chaperone structure–function and client recognition (see #3 below).

While I was passionate about in-cell folding, I also knew that principles of protein folding *in vitro* were critical to folding in the cell. The same rules governing conformational preferences of particular sequences would have to apply *in vivo* as well as *in vitro*. Many of the proteins whose folding had been studied intensively *in vitro* were rich in α-helices, the formation of which was governed to a great extent by local sequence and interactions among neighboring residues. When one of my students, Zhi-Ping Liu, came to me proposing that we examine the folding of aβ-barrel protein under study in Joe Sambrook's lab for non-folding related reasons, cellular retinoic acid-binding protein I (CRABP I), I was won over. Zhi-Ping, Patricia Clark, Jose Rizo-Rey and others did a great job of elucidating how this protein folded (see #4 below).

The move to UMass Amherst demonstrated how academic administration can suck up one's time. Happily, I had great trainees who kept things moving (Patricia, Ning Zheng, many others). And felicitously, my passion to delve deeply into folding in the cell rescued me at the end of my two stints as Department Head and convinced the NIH to award me one of the early Pioneer grants in 2006. This grant opened many doors as we could ask daunting questions, hire talented trainees in greater numbers than possible on an R01, and overall have a scientific blast.

As I've emphasized, my research directions and any significant accomplishments have been shaped by the people working in my lab... the trainees. Among those joining the lab during the five-year Pioneer grant period was Zoya Ignatova, who took on the challenge of developing a reporter system to monitor protein stability in cells, and the resulting sensor felicitously moved us into the area of protein aggregation (see #5 below).

We enjoyed enriching our love affair with Hsp70 over our early years at UMass Amherst, catalyzed by Diana Montgomery initially and culminating with an extremely fruitful elucidation of the Hsp70 allosteric mechanism via powerful NMR methods led by Joanna Feltham Swain and carried over the goal line by Anastasia Zhuravleva and Eugenia Clerico (see #6 below). Eugenia has continued leading our efforts to understand client recognition by Hsp70 in exquisite detail (see #7 below).

As I've alluded to above, collaborations have impacted and enriched our work in many ways. Meeting Evan Powers at a symposium in Japan, by chance, led to a delightful synergistic effort to computationally model the complex protein homeostasis (now known as 'proteostasis') networks in *E. coli*, and the program "FoldEco".[4] Our joint effort was made possible by Evan's ability to harness mathematical approaches to coupled differential equations. We have continued to stay in close touch, and our teams developed experimental tests to validate the predictions of FoldEco.[5,6]

More recently, my lab has joined up with the highly productive and uniquely expert lab of the late Dan Hebert, a close friend with whom I worked closely over the last 27 years and who died well before his time this past December. For me, this has been a "full circle" kind of collaboration, as Dan's lab has focused on proteins that harbor signal sequences targeting them to the endoplasmic reticulum in eukaryotic cells. The majority of these proteins are N-glycosylated, and Dan's work has revealed the importance of the glyco-proteostasis network in proper folding and quality control of the "secretome". Our complementary expertises have enabled exploration of structural, biochemical and cell biological aspects of the glycoproteostasis network (see #8 below).

While my own work has not yet moved into this arena, it is clear that the field of proteostasis is now front and center in therapeutic approaches to many diseases.[7] A couple of major successes that illustrate the potential of therapeutic strategies based on proteostasis are the amyloidosis therapy, tafamidis,[8] and the modulator of cystic fibrosis transmembrane receptor localization and function from Vertex, Trikafta.[9] It has been truly eye opening to see the transition from basic science to biomedical therapies.

Research Stories Im Particularly Proud of

1. *Cyclic peptides as models for reverse turns.* I will never fall out of love with cyclic peptides. These highly conformationally constrained polypeptides serve many purposes. My own were to build well behaved models for β- and γ-turns. This led to the central paper coming from my doctoral thesis.[10] Later, as I started my position at Amherst College, we made the huge jump to cyclic pentapeptides (not a huge jump. . .) and developed models that illustrated features of both γ − and β-turns (Figure 2). A chance encounter with Isabella Karle in the women's room of the Barbizon Plaza Hotel during a New York Academy of Sciences meeting (interestingly focused on women in science) enabled the side-by-side publication of crystal structures from Isabella's work of many of the cyclic pentapeptides we designed and studied in solution[11,12] and others reviewed in[14]). A wonderful period ensued, in which these well-defined peptides became useful tools for many developmental experiments, notably solid-state NMR studies spearheaded by our collaborator Stan Opella, then at UPenn.[14] He and I maintained a close collaboration until his UCSD move and his increased focus on membrane-interactive peptides.
2. *Signal sequences.* Beginning with the work catalyzed by my encounter with Tom Silhavy, we used peptides encompassing signal sequences in many years of productive study. This work using synthetic peptides as models for signal sequences was justified because signal sequences could be moved from one secreted protein to another without disrupting their ability to target their protein "passenger" correctly, arguing that their intrinsic properties were necessary and sufficient for targeting. Thus, we characterized wild-type and mutant signal sequences and sought an understanding of the biophysical properties that conferred on them the ability to shepherd the passenger to the export/secretory machinery. The first set were the LamB signal sequences Emr and Silhavy had postulated must adopt an α-helical conformation to be functional.[2] Indeed, the conformational propensities of the signal peptides supported their hypothesis (Figure 3). We partnered with Bill DeGrado and Jim Lear to show how these and other synthetic signal peptides spontaneously inserted into membranes,[15] later enhanced by Don Cornell's ability to show that they adopt an α −helical conformation when inserted,[16,17] and to correlate the loss of these abilities in mutant versions with their degree of loss of function (Figure 4)[18]. This work, while emphasizing a highly simplified system, yielded insights that have

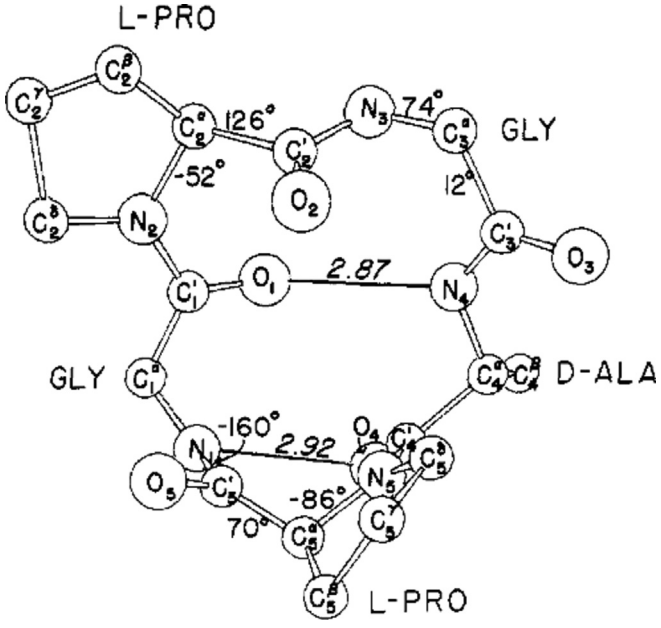

Figure 2. Crystal structure of the cyclic pentapeptide, cyclo-(Gly-Pro-Gly-D-Ala-Pro)[11]. The structure of this cyclic pentapeptide is the same in solution[12,50] as in the crystal and is made up of a β-turn (corner residues Pro-Gly) linked to a γ-turn (encompassing D-Ala-Pro-Gly).

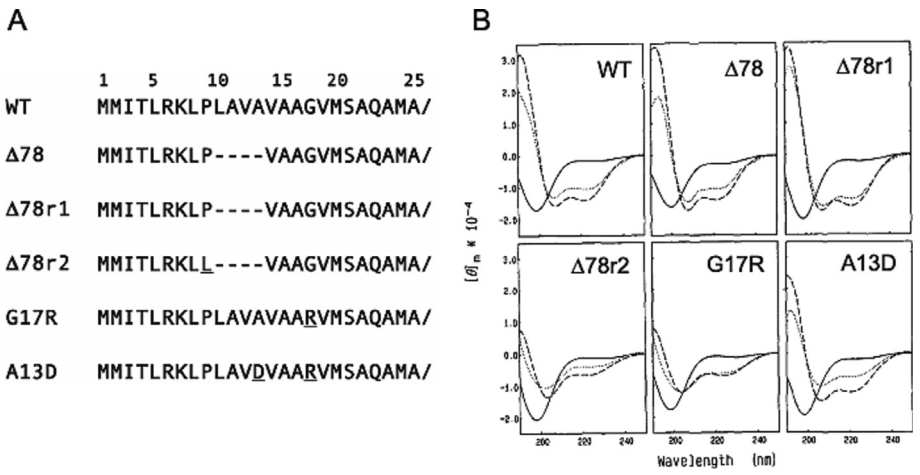

Figure 3. A. Sequences of LamB signal sequences synthesized as peptides. CD spectra of the corresponding signal peptides in aqueous buffer and in SDS micelles are shown in B. The relative functions of these signal sequences in an assay for export of LamB to the outer membrane of *E. coli* are as follows: WT 100%, Δ78 0%, Δ78 r1 50%, Δ78 r2 90%, G17R 40%, A13D 10%.[51] Note that the ability of these sequences to adopt an α-helical conformation in membrane-mimetic environments parallels their ability to function in targeting LamB to the outer membrane. Figure from.[18]

stood the test of time and fit beautifully with the increasingly sophisticated knowledge about the more complex targeting apparatus and mechanism coming from work of many labs.

3. *Chaperones: GroEL/ES, Hsp70.* In the early days of chaperones, we wondered how these helper proteins interacted with a wide array of clients but discriminated between those that were natively folded and those that were incompletely folded, and whether there were differences in how the chaperonins, with the bacterial chaperonin GroEL serving as the best example at the time, and the Hsp70 family, with the *E. coli* family member DnaK as the best example, accomplished this. My lab deployed NMR as a primary technique, but GroEL and Hsp70 were large enough to make this approach tough. And we loved making peptides. So we exploited a method (measurement of transferred nuclear Overhauser effects, tr-NOE's) that allowed the conformation of a smallish molecule to be revealed by the pattern of tr-NOE's it developed when bound to a large molecule. In this way, we saw that the same model peptide bound DnaK in an extended conformation and GroEL in a helical conformation (Figure 5).[19] While the detailed understanding has expanded greatly, our initial observation was key to early appreciation of chaperone/client binding and, most impressively, it was obtained before we had the structures of these chaperones to look at. Along similar lines, Sam Landry came to me and said he wanted to do NMR on GroES, the partner co-chaperone of GroEL. I attempted in vain to dissuade him, and the result was a superb paper[20] in which Sam identified a mobile region of GroES (hence, NMR observable) that became immobilized when its complex with GroEL formed, supported the importance of this region by looking at Costa Georgopoulos genetic data, hypothesized before any structures were available that a conserved tripeptide hydrophobic sequence in the mobile region of GroES was involved in GroEL binding, and pointed to the generality of these observations (Figure 6). The "mobile loop" of GroES should really be called the Landry loop!
4. *Folding of a β −barrel protein.* When we launched our project on the folding of CRABP 1, we recognized that folding of β-rich proteins was less well understood than that of primarily helical proteins. Moreover, CRABP 1 adopts a structure with a central cavity and closure of β-strands around the cavity in a barrel stabilized by hydrogen bonding (Figure 7A, B). The concept of a molten globule was prevalent in the folding field but most readily fit to a helical protein, in which secondary structure could be sampled with some sequestration of hydrophobic groups. How this would apply to CRABP 1 and similar proteins and whether their folding intermediates were fundamentally different was a puzzle. Enter Zhi-Ping Liu, Jose Rizo-Rey, and with the capstone experiments, Patricia Clark. These lab members showed in a series of experiments that CRABP 1 folded through a series of intermediates, with early collapse, then formation of the barrel and its cavity but not stable hydrogen bonding and tight packing, which developed only in the last folding step (Figure 7C).[21–25] The presence of topology in the penultimate intermediate gave us a model for a β-molten globule.
5. *In-cell folding and aggregation.* We aspired to move our study of protein folding into the cellular environment, and to do so we designed a reporter based on Roger Tsien's FlAsH fluorescence system. Zoya Ignatova, Beena Krishnan and others in the team

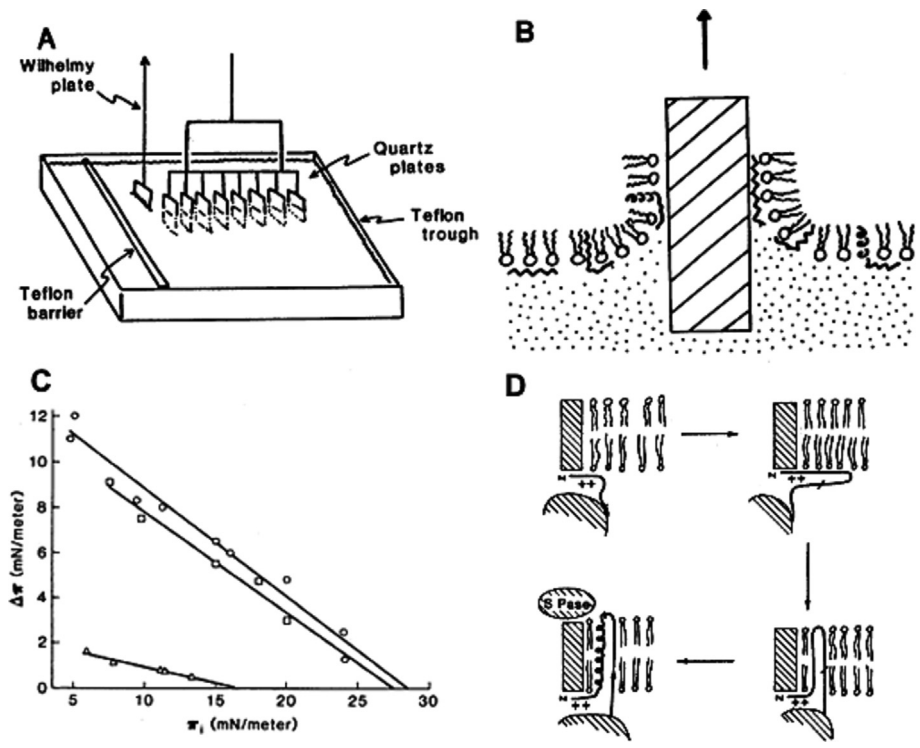

Figure 4. A. The ability of LamB signal peptides to insert into a lipid monolayer correlates with the function of the corresponding signal sequences in targeting their passenger to the outer membrane of *E. coli*. Plotted is the change in surface pressure ($\Delta\pi$) as a function of initial surface pressure (π). The wild-type signal peptide (plotted as circles) and a functional revertant (plotted as squares) both insert against higher surface pressures than the peptide that corresponds to a non-functional signal sequence (plotted as diamonds). B. Apparatus that was used to transfer phospholipid monolayer samples containing signal peptides onto a solid support for spectroscopic analysis. The use of a trough to maintain a desired surface pressure, monitored by the Wilhelmy plate, enabled characterization of signal peptide conformations as a function of surface pressure. C. Conformations observed at different surface pressures in the transferred monolayer pointed to the ability of the signal peptide to adopt β-structure at the surface when pressure is high and to undergo a conformational transition to α-helix upon insertion, leading to a model for signal peptide function *in vivo* (panel D). Panel A is from[15] and panels B, C and D are from.[16]

found a way to incorporate the FlAsH reporter into the protein whose folding we had studied most deeply *in vitro*, CRABP 1 (Figure 8A).[26,27] In addition to measuring denaturant unfolding of this protein in *E. coli* (which tolerate up to 3 M urea) (BFigure 8B), this system revealed itself to be a great way to follow protein aggregation in cells (Figure 8C).[28] Zoya took this one step further and used this reporter to demonstrate how proline could act as an osmolyte inhibitor of aggregation[29] and to reveal mechanistic aspect of the aggregation process including as a target of study a fusion with exon 1 of Huntingtin.[30,31] We understood well that there were limitations to this reporter and the general approach, but nonetheless it illustrated how an engineered reporter might open doors into folding and aggregation in the in-cell world.

6. *Hsp70 allosteric mechanism.* While we worked on the GroEL/ES system for some time after our earliest ventures, we increasingly focused on Hsp70s. I am grateful to Diana Montgomery and Ed Feng for really pushing this work early on. And along came Joanna Swain and later Anastasia Zhuravleva and Eugenia Clerico, who with others in the lab worked out the allosteric mechanism of DnaK using primarily NMR.[32–36] Their work anticipated the structural insights that were slow to come, because the ATP-bound state of DnaK was crystallographically elusive for some time.[37,38] Moreover, in deploying NMR, they could see the allosteric landscape of this wonderful machine and demonstrate how interdomain interfaces poised it to respond allosterically to ligand binding (Figure 9). We reveled in our understanding of how the simple addition of the γ-phosphate of ATP could trigger a massive conformational change in DnaK accompanied by increased catalytic activity and decreased affinity for substrates.[34,39] Hydrolysis of nucleotide to ADP in turn caused the chaperone to take up a conformation with its two major domains, the substrate-binding domain (SBD) and the nucleotide-binding domain (NBD), to dissociate from one another, reduce the on/off rates of substrate binding, and lead to a higher substrate affinity. This allosteric conformational change, modulated by co-chaperones, enables Hsp70s to act as two-stroke

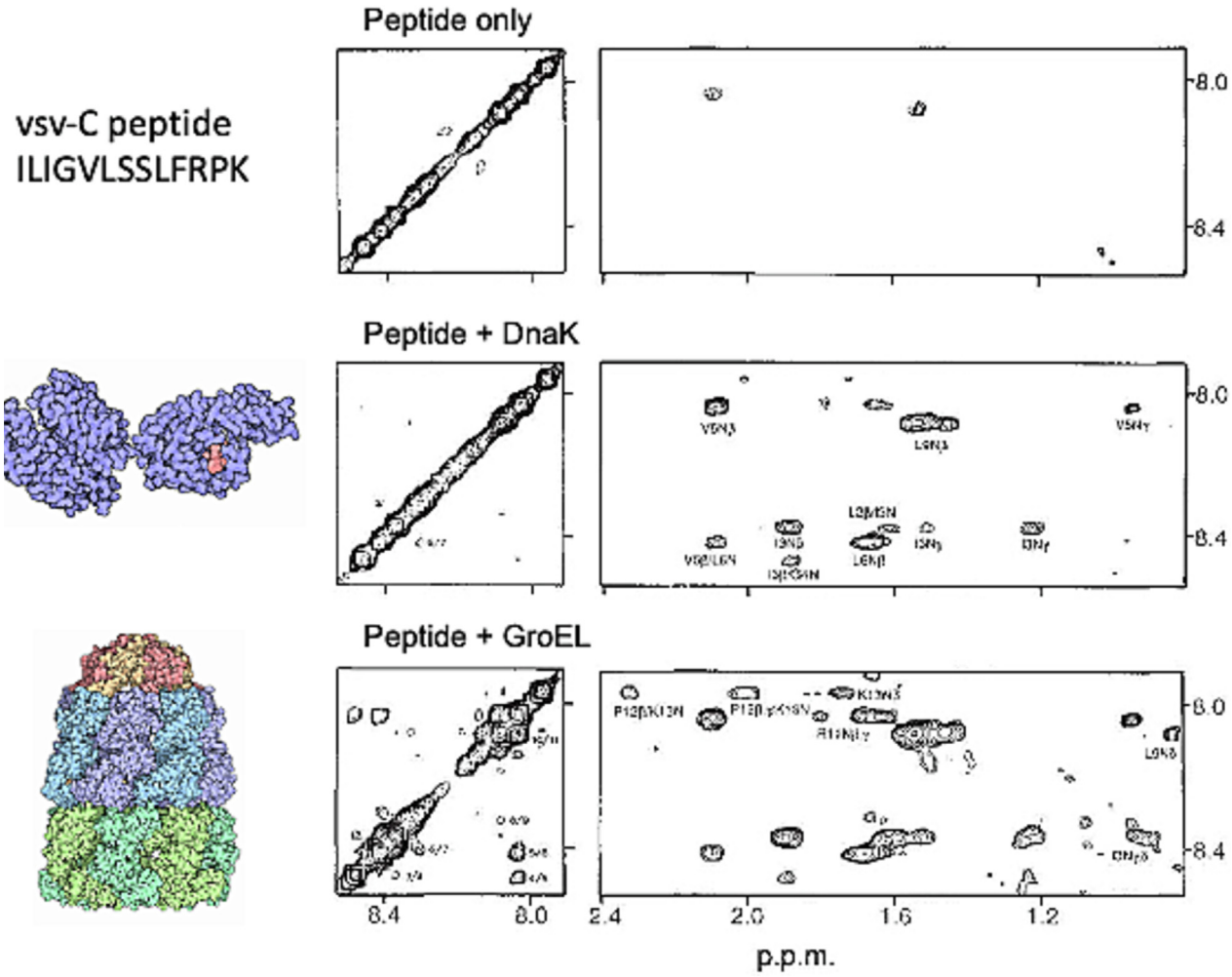

Figure 5. Binding of the same peptide to DnaK or GroEL revealed that these two chaperones utilize different modes of client interaction. Two distinct bound conformations are indicated by trNOEs observed upon addition of chaperone to a solution of the vsv-C peptide. Particularly informative were the NOEs diagnostic helical conformation (NH-NH and NH to Hα i + 3 and i + 4) observed in the GroEL-bound peptide and absent in the DnaK-bound peptide. Figure from.[19]

machines, binding and releasing their substrates at a rate that is governed by their intrinsic structural features, their interactions with co-chaperones, and the extant ratio of ATP to ADP.[40]

7. *Hsp70 substrate binding.* In recent years in the lab, we have delved with increasing depth into the nature of substrate (client) binding to the SBDs of Hsp70s. We have leaned heavily on NMR, although partnering NMR with x-ray and with biochemical approaches to determine affinities and orientation of bound substrate models has been invaluable.[41] The fun aspect of this chapter in our research is that we keep learning more! At the beginning, we assumed that the canonical orientation of substrates first seen in the landmark crystal structure from Zhu and Hendrickson[42] would obtain for most polypeptides bound to DnaK (Figure 10A and B). We recognized that the opposite orientation had been seen in some structures,[25,43] but most of the field built their models of bound substrates assuming they were oriented as in the early crystal structure. Several exciting steps later we know that both orientations can occur and that they are in fact close in energy, in large part because an unrequited β-strand in the SBD can partner with the substrate in either orientation (Figure 10C). We also have a much better understanding of how some selectivity is achieved in Hsp70 binding to its clients, giving preference to incompletely folded states, but how the ability to interact with a large fraction of the proteome (promiscuity) is achieved. The hero in this story, after Eugenia Clerico, Rachel Jansen, and Alexandra Pozhidaeva, is methyl NMR. The chemical shifts of two isoleucine $\delta-$methyls that line the substrate binding site of DnaK and happily Hsc70 (HspA1 has only one) are exquisitely sensitive to the identity of the central residue in the sequence of five that make up the most important binding region of the substrate and to the orientation of the bound substrate (Figure 10D). The insights this striking correlation has enabled us to derive from this study have helped the field better understand the key role of Hsp70s in client recogni-

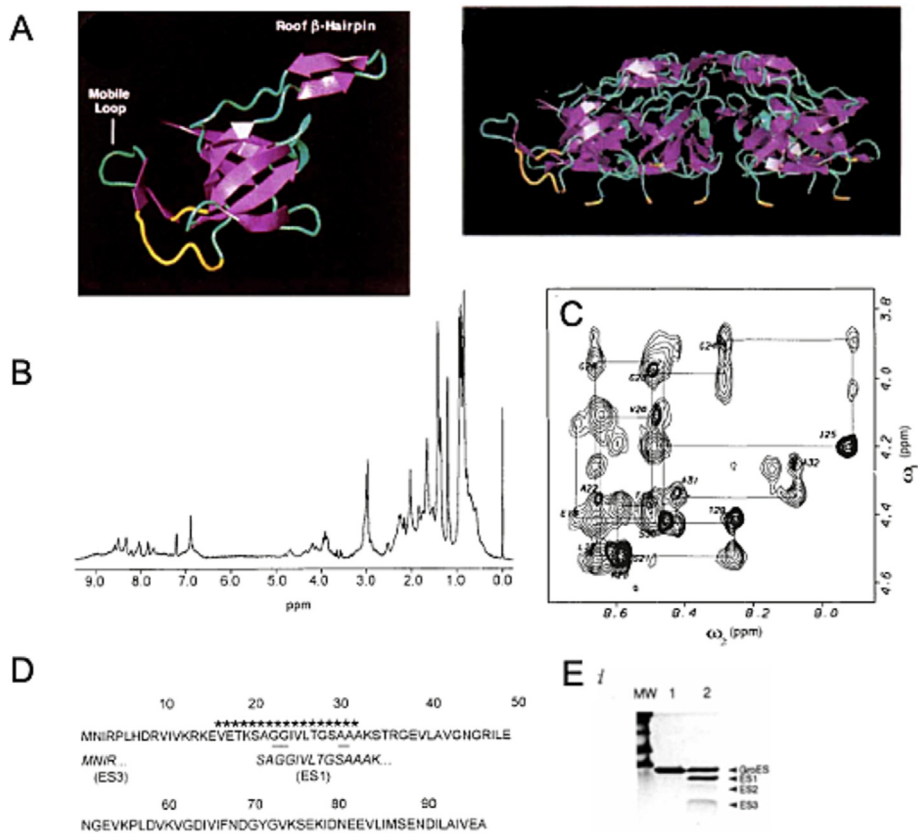

Figure 6. GroES has a mobile region that is the site of binding to GroEL. A. The one-dimensional NMR spectrum of GroES revealed a set of narrow resonances that could be sequentially assigned by two-dimensional homonuclear NMR (B) and associated with a sequence in GroES (C) that is highly dynamic. C. Supporting the dynamic character of this region are limited trypsinolysis experiments showing that GroES is cleaved at one primary site. D. In x-ray crystallography (carried out after the NMR work) the mobile loop is unresolved in all but one copy of the GroES heptamer and is oriented towards the GroEL interaction surface. By NMR, the mobility of the loop was strongly reduced upon addition of GroEL to the sample. Panels A, B and C are from[20] and panel D is from.[53]

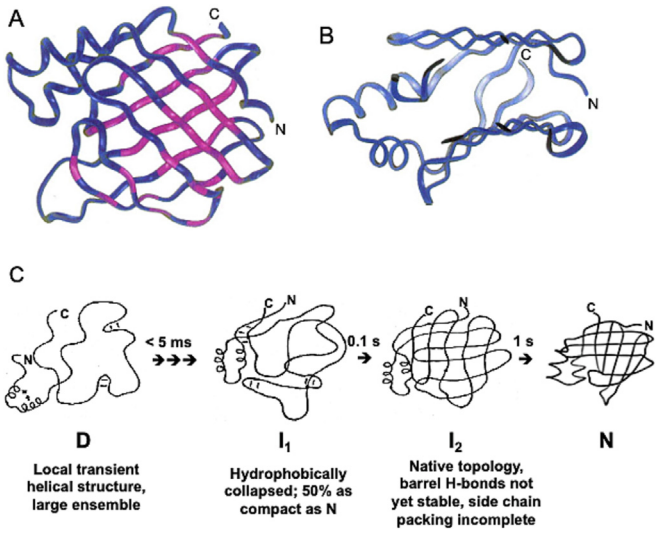

Figure 7. A. The structure of cellular retinoic acid binding protein 1 (left top view, right side view) with the NHs that are slowly exchanging with D_2O.[21] The hydrophobic cavity used to bind retinoic acid is visible in the side view. B. The kinetic mechanism of folding of CRABP 1 derived from fluorescence, NMR and CD data.[21,22]

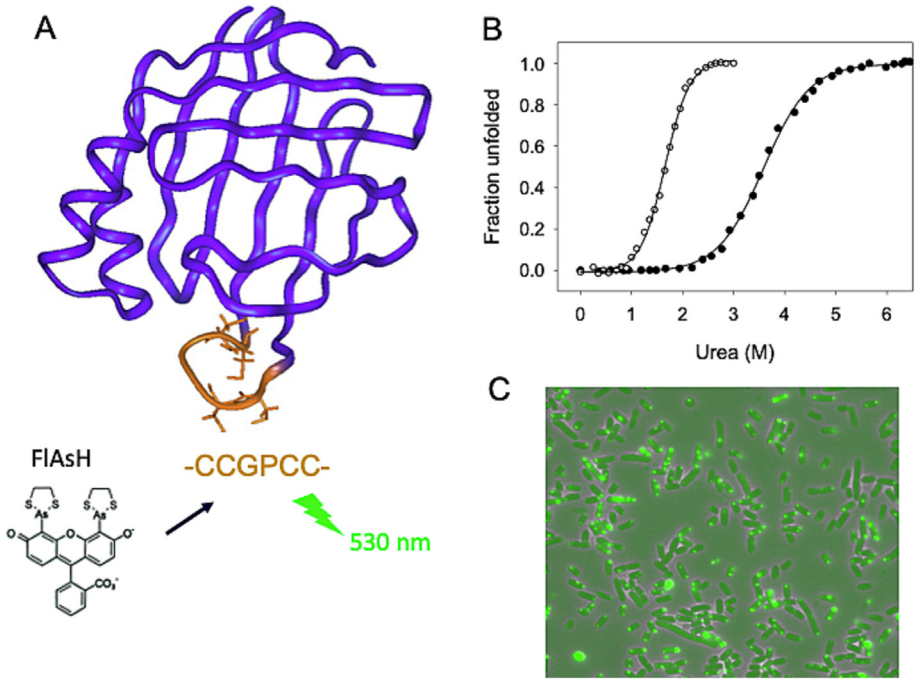

Figure 8. A. Design of a FlAsH-binding variant of CRABP 1. The four FlAsH-binding cysteine residues were introduced into a loop in the protein that has relatively low sequence conservation and is dynamic. B. Urea melt of FlAsH-labeled CRABP 1 expressed in *E. coli* cells. C. Fluorescence micrograph showing the bright fluorescence emanating from largely polar localized aggregates of CRABP 1. Reproduced from.[26]

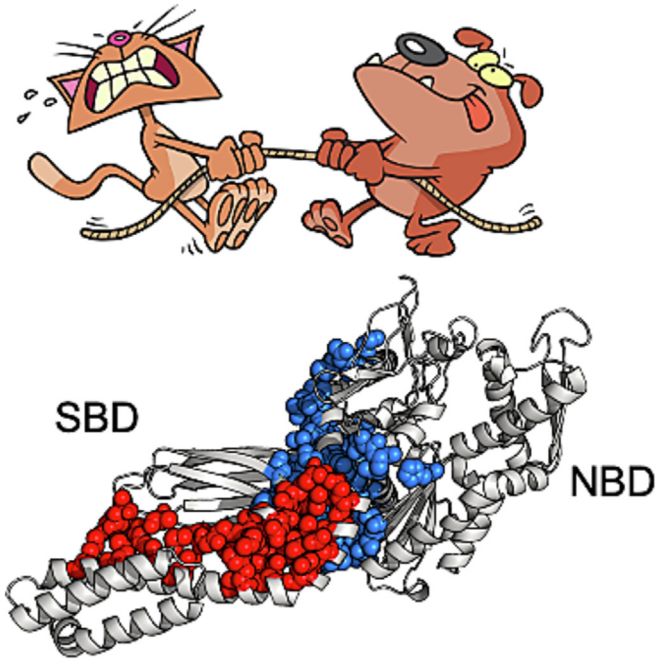

Figure 9. The allosterically active state of DnaK populated when both ATP and a substrate peptide are bound. ATP favors the fully docked arrangement of the NBD and SBD and formation of an intimate interdomain interface (blue), but substrate binding favors domain dissociation and the high affinity state of the SBD, with the interface between the helical lid and the β-subdomain (red). The energetic competition created between these two ligand-favored interfaces[34] is schematically shown as a tug-of-war between a dog and a cat.

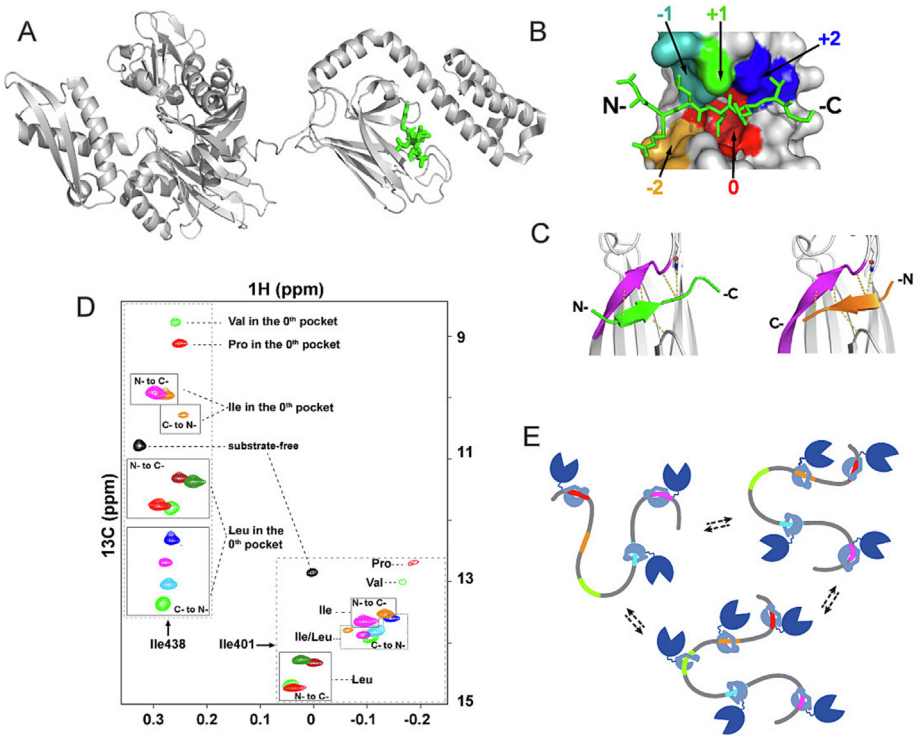

Figure 10. A. The site of substrate binding to DnaK is shown in the structure in green (PDB 1DKZ). B. Top view of the substrate-binding site after removal of the α-helix, showing the pockets for substrate side chain interactions. The peptide illustrated is NRLLLTG, and the orientation in this structure is what we call N- to C-, or forward. C. Schematic illustrations of N- to C- (forward) and C- to N- (reverse) binding, showing that one is stabilized by a parallel β −sheet association and the other by an anti-parallel β −sheet. D. HMQC spectra of δ1-methyl ^{13}C-labeled Ile in the SBD, showing the diagnostic chemical shifts upon binding of model substrate peptides with different residues occupying the 0th pocket position or in different orientations. E. Schematic of DnaK binding to an unfolded protein, indicating that multiple chaperones can bind at preferred sites in a fluctuating manner governed by the ratio of nucleotide, ATP/ADP. Panels D and E are reproduced from.[41]

tion and how this two-stroke engine can bind and release at sites along a client to modulate its folding and inhibit its aggregation (Figure 10E).

8. *Folding in the endoplasmic reticulum (ER) and glycoproteostasis.* With heavy heart, I describe one of the most fun and informative collaborations I've had in recent years, made possible by the arrival at UMass Amherst 27 years ago of my recently deceased dear friend and colleague, Dan Hebert. Dan's lab has become world leaders in how protein folding and quality control occur in the lumen of the ER.[44] One third of the mammalian proteome traverses the ER *en route* to be secreted, trafficked to the plasma membrane, or localized to other non-cytoplasmic destinations. These proteins are targeted by carrying a signal sequence, which was perhaps the glue that first brought Dan's and my programs together. In addition, we were joined by a visiting faculty member Anne Gershenson (now at NIGMS), who is an expert on serpins and how their misfolding and aggregation lead to pathologies. Together we have spent a fruitful time using serpins as model secreted proteins to gain insight into their folding in the ER.[45–48] The collaboration between my lab and Dan's has grown over the last few years such that members of my group began to apply biophysical strategies to better understand proteostasis in the ER. We thus have had a ringside seat as Dan and his team discovered the way N-glycan handles on secreted proteins serve to guide them through the network of ER chaperones in such a way as to facilitate their folding and quality control: glycoproteostasis.[44,48] A postdoc shared between our labs, Rob Williams, has been focusing on the gatekeeper in ER glycoproteostasis, UDP-glucose: glycoprotein glucotransferase (UGGT) (Figure 11).[49] As we work to see the last major contributions from Dan's lab published, we are seeking to make inroads into the structure–function relationships and client recognition by UGGT.

Overview and Future Directions

It is stunning to consider how the field of protein folding has evolved during my career. We now happily draw three-dimensional funnels and energy landscapes to illustrate the process of protein folding, and we have the ability to predict structures with unprecedented success. Yet it remains one of the challenges of biophysics to truly understand the mechanism of folding. Moving

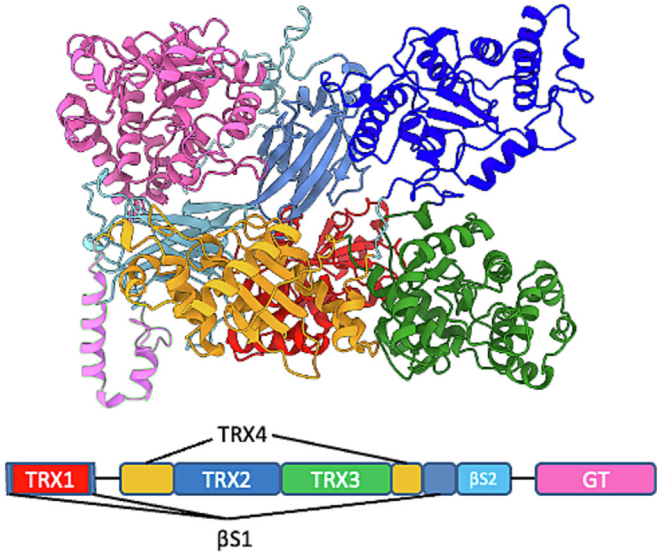

Figure 11. Top: AlphaFold2 prediction of the structure of the human ER folding/quality control gatekeeper, UGGT,[49] with domains colored as indicated on the schematic below, as follows: TRX 1, 2, 3 and 4 are thioredoxin homologous domains; β S1 and β S2 are two β-rich folds; and GT is the glucosyl transferase that modifies clients. In pink is the region predicted to bind the co-chaperone, Sep15. This region is only present on UGGTs from organisms that have this selenoprotein as a component of their ER proteostasis network.

this understanding into the context of the cell, with folding initiated while a polypeptide is translated on the ribosome and is being targeted to its proper cellular destination, presents even more challenges and mysteries. We have gained a great deal of knowledge about these processes, but many gaps remain. Moreover, we continue to rely heavily on simplified systems, either purified or reconstituted with some retention of the cellular components. . .but certainly these systems are very different from the crowded, complex, multi-component, regulated, dynamic, organized environment in which biology occurs. I believe a Holy Grail in understanding the biophysics of protein folding is to be able to explore this process *in situ*. Cryo-electron tomography and cryo-electron microscopy are helping greatly and show promise of providing views into the cellular world. However, we still need to develop more methods to take these views in the cellular world and follow folding and targeting as they occur, including their dynamics and their modulation by post-translational modifications, etc. Relating the insights that will be gleaned to pathologies holds promise of new approaches to therapeutics. We have seen increasing numbers of diseases with etiology arising from protein misfolding. Neurodegenerative diseases and prion diseases are obvious examples, but many more are being discovered, and most are made more probable due to aging. How these are linked is not yet clear, but I am confident the next years will give us amazing new understanding. For those coming along in their careers, please be bold and tackle these research challenges! They will require methodological advances, and through these advances, discoveries will be made that fill current knowledge gaps and open our horizons to new and ever more exciting challenges.

My parting thought is that while scientific discovery is incredibly rewarding, the people one encounters through science represent an overriding source of joy. I have made such great friendships through collaboration and collegialism. And for me, working with trainees and seeing them blossom into colleagues has been the most satisfying aspect of my career. If in any small way I have facilitated the growth and encouraged the passion of my trainees, then my career efforts have been worthwhile. I close with a photograph from a Gierasch lab reunion held right before the COVID pandemic would have stymied it, in 2019 (Figure 12). My past trainees put together a book for me with messages about the impact of their time in lab on their lives and career paths. I used up a whole box of Kleenex reading it.

CRediT authorship contribution statement

Lila M. Gierasch: Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Figure 12. Gierasch lab reunion, 2019. Picture was taken by a small personal drone, handled by Zhi-Ping Liu.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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