Membrane protein serendipity

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My scientific career has taken me from chemistry, via theoretical physics and bioinformatics, to molecular biology and even structural biology. Along the way, serendipity led me to work on problems such as the identification of signal peptides that direct protein trafficking, membrane protein biogenesis, and cotranslational protein folding. I've had some great collaborations that came about because of a stray conversation or from following up on an interesting paper. And I've had the good fortune to be asked to sit on the Nobel Committee for Chemistry, where I am constantly reminded of the amazing pace and often intricate history of scientific discovery. Could I have planned this? No way! I just went with the flow . . .

What is life but a succession of chance events and poorly informed decisions? It is only in hindsight that we can dream up a life story with even a hint of logic planning and intelligent foresight. This makes Reflections hard to write—if you don't have an exhilarating story of success against all odds or something of that ilk, you start to worry that your scientific career might sound like some ramblings about more or less random encounters and happenstance discoveries. Yet with advancing age comes an urge to reminisce, to summarize, to put in order, to distribute credit (and sometimes blame); in short, to reflect on life. And a hope that other people will even enjoy reading such texts. My assumption is that you are one of these people, lest you would not have read this far, and my best excuse for writing this piece is that I've had fun doing it ;-).

So, can I tell you anything interesting about myself as a person? Not much, except perhaps that I hate to dress up, and always have. My one traumatic childhood memory is when in kindergarten I was forced to dress up in a Lucia costume—for some odd reason, every year on December 13, Swedes celebrate the butchering in A.D. 310 of the Sicilian Saint Lucia by putting white robes on the kids, with the girls having candles in their hair and the boys wearing white dunce caps with gilded paper stars on them. I absolutely refused to take part, and spent the afternoon crying on the sidelines. A defining moment, if ever there was one. One that I've never been able to free myself from, it seems, as even now I feel uncomfortable wearing anything but T-shirts (Fig. 1) . . .

After that auspicious beginning, here's the rest of my biography: Master in Chemical Engineering at the Royal Institute of Technology (KTH) in Stockholm in 1975, Ph.D. in Theoretical Physics at KTH in 1980 (with Clas Blomberg), postdoc at the University of Michigan in 1981 (with Mike Savageau), Assistant Professor at KTH (1981–1987), Associate Professor at Karolinska Institutet (1988–1994), and Professor at Stockholm University (1994 to today).

Why this particular career path? Chemistry, because I got a stipend from my chemistry teacher in high school to attend the Berzelius Days, an annual event where the Swedish Chemical Society tries to interest kids in chemistry (the quantum chemistry lecture was particularly exciting, as I recall). Theoretical physics, because the professor who taught physical chemistry at KTH told me that "those physics guys are the real stuff, chemists are just poor copy-cats." The University of Michigan because, while still a Ph.D. student, I met Mike Savageau at a summer school in Warsaw (in the days when there was only one phone in the whole of Warsaw that you could call home from). Karolinska Institutet, because when spending some time in Bill Wickner's lab at UCLA (more below), I happened to see an ad on a poster board announcing that Henrik Garoff—who had just joined the Karolinska as a professor of molecular biology—was looking for new staff. I jumped at the chance, since I was just thinking about how to make a move from bioinformatics toward wet-lab biochemistry. Stockholm University, finally, because the then head of the Biochemistry Department, Bertil Andersson (who later became President of Nanyang Technical University in Singapore), called me out of nowhere and suggested that I apply to a newly advertised professorship in Theoretical Chemistry—a position I was convinced would go to a quantum chemist, not a bioinformatician with wet-lab ambitions. Fortunately, the recruitment committee thought otherwise.

OK, with these preliminaries done, let's talk science. It all started with a totally serendipitous (and super-important) event: through no fault of my own, I stumbled across the famous 1975 Blobel and Dobberstein paper where they reconstituted protein translocation across the ER[2] membrane *in vitro* and proposed the Signal Hypothesis (1) (*i.e.* that proteins contain short sequences that serve as signals to direct their transport around the cell, work for which Günter Blobel was awarded the Nobel Prize in Medicine or Physiology in 1999).

I was still a Ph.D. student at the time, and had taken advantage of an offer by KTH to take a free language class in French.

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[2] The abbreviations used are: ER, endoplasmic reticulum; TMH, transmembrane helix; AP, arrest peptide.

Figure 1. No Lucia costume for this little guy.

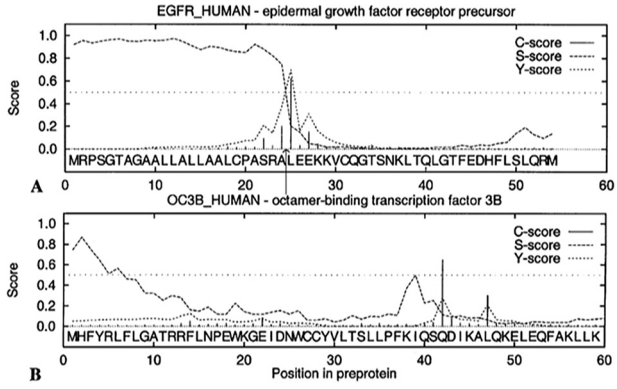

Figure 2. Typical outputs from SignalP 1.0 (5). The sequence in *panel A* has an N-terminal signal peptide (correctly predicted by the high *S*-score) that is cleaved between Ala^{24} and Leu^{25} (correctly predicted by the high *Y*-score). The sequence in *panel B* is a cytoplasmic protein that lacks a signal peptide. Reprinted from Protein Engineering. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering*. 1997; 10:1–6. © the Oxford University Press (5).

This led me to subscribe to the French popular science magazine *La Recherche*. One day, I read a short piece on something called "signaux d'adressage" (*i.e.* signal peptides). An accompanying figure didn't make sense to me, and out of curiosity I looked up the original Blobel and Dobberstein paper. Wow! Protein translocation looked like something I, as a theoretician, could try to model! I read up on what little was published at the time about hydrophobicity as applied to peptide–lipid interactions, and tried to estimate the energetics of moving an unfolded polypeptide chain across a lipid bilayer (2, 3). Of course, we now know that the polypeptide chain of a secretory protein is translocated across the ER membrane through a proteinaceous channel, the Sec61 translocon, rather than directly through the lipid bilayer, so, as it turned out, this analysis didn't exactly address the question at hand. However, it came in handy later when I became interested in membrane proteins.

At any rate, Blobel somehow got wind of my rather naive modeling paper, and I was invited to a now classic meeting on Membrane Biogenesis in Cold Spring Harbor in 1979—the first time ever I visited the U. S. All the pioneers were there: Günter Blobel, David Sabatini, Bernhard Dobberstein, Jon Beckwith, Bill Wickner, and more. Not that I had any idea back then what kind of select company I found myself in, but it was quite an experience. And it encouraged me to stick with protein translocation and, eventually, membrane protein biogenesis.

But I was in a theoretical physics department. How could I make any kind of useful contribution to what was essentially a cell biology/genetics field? Again, I was lucky: cDNA sequencing was just coming on-line, and suddenly scores of sequences of signal peptides could be obtained "for free" from the DNA sequences, rather than by laborious peptide sequencing schemes. A couple of weeks in the library (remember the flimsy paper of the Science Citation Index tomes?), and I had almost a hundred signal peptide sequences to analyze. What came out of this was a first attempt to make a predictor to identify the cleavage sites for the signal peptidase (the enzyme that cleaves signal peptides from the intact protein) (4). Some years later, together with the machine-learning gurus Henrik Nielsen, Jacob Engelbrecht, and Søren Brunak at the Danish Technical University, we developed the widely used SignalP predictor (5) to predict signal peptides and their cleavage sites (Fig. 2). SignalP is still being perfected (6–8), and version 5.0 is now in the works.

What do we know today about signal peptides? First, that there are many more kinds than the initial ones that were identified for targeting proteins to the eukaryotic ER or the bacterial inner membrane: we now know about mitochondrial targeting peptides, chloroplast transit peptides, peroxisomal targeting sequences, nuclear localization signals, etc. Moreover, there are closely related siblings of the original signal peptides that mediate the translocation of lipoproteins or the secretion of fully folded proteins (the twin-arginine translocation pathway) across the bacterial inner membrane. All of these different signal peptides can now be predicted in amino acid sequences with a fair success rate (9), and a lot is known about how they are recognized by cellular targeting-and-translocation machineries (10).

If signal peptides happened to have been my entry point into bioinformatics (although the term "bioinformatics" had not yet been invented back then), what really became my main obsession was membrane proteins. From a bioinformatics perspective, this was just a small step, since signal peptides are composed of a central hydrophobic segment flanked on the N-terminal end by a short stretch of positively charged residues—and the first few sequences of integral membrane proteins that were published around 1980 contained segments with very similar characteristics (11). In fact, the flanking

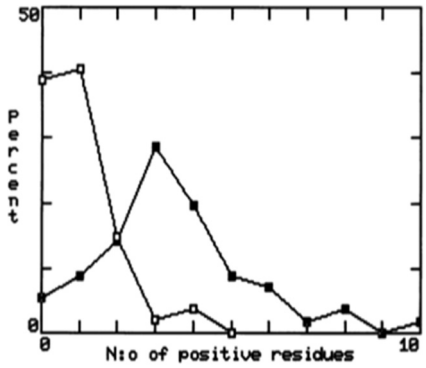

Figure 3. The positive inside rule. The plot shows the percentages of all cytoplasmic or periplasmic loops in a collection of bacterial inner membrane proteins that contain a given number of Arg + Lys residues: positively charged residues are more prevalent in cytoplasmic loops (*black squares*) than in periplasmic loops (*white squares*) (12). I was particularly proud of this figure, since it was produced on a line printer that I had programmed to print plots (so I wouldn't have to draw them by hand in India ink). Reprinted from EMBO Journal. von Heijne, G. The distribution of positively charged residues in bacterial inner membrane proteins correlates with the trans-membrane topology. *EMBO J.* 1986; 5:3021–3027. © John Wiley & Sons, Inc. (12).

charged residues turned out to be key: even a cursory look at the few membrane proteins with known topology that I could gather from the literature in the late 1980s showed that the positively charged residues were found mainly in the parts facing the cytoplasmic side of the membrane (12) (Fig. 3). This eventually became known as the "positive inside rule" (a professor in the theoretical physics department convinced me that inventing a catchy term was the best way to market one's research . . .).

One neat thing about the positive inside rule was that it suggested a simple way to improve the then existing algorithms for membrane protein topology prediction (13): identify those segments in a membrane protein that have borderline hydrophobicity, construct all possible topologies where those segments are either assumed to be transmembrane or not, and score the topology models according to the bias in the distribution of positively charged residues between the two sides (Fig. 4). This scheme significantly boosted prediction performance over older methods that did not take the positive inside rule into account. Of course, modern methods using machine learning but built on the same basic idea perform even better (14); today, topology prediction is a fairly mature field in membrane protein bioinformatics.

A year or so after coining the positive inside rule, I decided to take a short sabbatical with Bill Wickner at UCLA that also turned out, quite unexpectedly, to have a major impact on my science. Because our kids were still young enough to take abroad for a semester, my wife and I decided that spending a winter in L. A. would be a good idea. Bill was turning 40 (his friends gave him a gorilla suit for his birthday), and his lab had just discovered trigger factor (a protein now known to be the first chaperone that meets a nascent polypeptide as it comes out of the ribosome) and leader peptidase (Lep, the peptidase that cleaves signal peptides from secretory proteins). When I arrived with my "laptop" computer (I carried an early Macintosh in a very heavy shoulder bag), he looked a little wary and suggested that "I do something different for a change," namely get my hands dirty in the laboratory. I was still thinking about the positive inside rule, and Ross Dalbey in Bill's lab was doing site-directed mutagenesis on Lep. The idea was not hard to come by: test the role of positively charged residues for membrane protein topology using Lep, a protein with two transmembrane helices and a short cytoplasmic connecting loop full of Arg and Lys residues. Ross taught me the Kunkel method (40) for site-directed mutagenesis—laborious and reeking with ^{32}P—and how to label proteins with ^{35}S. This not only led to three pairs of blue jeans made radioactive by spilled samples, but eventually to the demonstration that Lep could be "turned on its head" in the membrane by relocating positively charged residues from the cytoplasmic loop to the protein's N terminus (15) (Fig. 5).

This happy discovery pushed my research in a direction that I couldn't have foreseen even six months earlier, and also pushed me to leave KTH and the Department of Theoretical Physics for Karolinska Institutet (KI) where I could continue with wet-lab work. Henrik Garoff from the European Molecular Biology Laboratory (EMBL) had just become professor of molecular biology at the new KI campus in Huddinge in Stockholm's southern suburbs, and was looking for new faculty. This was ideal: a new department with lots of empty space and sufficient funding so that I could set up a wet lab without having a big grant to support me. In fact, thanks to Jan-Åke Gustafsson, who was the prime mover behind the KI activities in Huddinge, money was so plentiful in those early years that when I eventually got my own funding, I had serious problems spending it. A once-in-a-lifetime experience . . .

The move to Huddinge made it possible to go after the problem of how membrane protein topology is controlled in more detail. IngMarie Nilsson, whom I could employ on my first grant, became a key person in this work (we're still colleagues, but now at Stockholm University). This grew into a major research program not only in our lab but in other labs as well, and the topological sequence determinants (charge, hydrophobicity) that guide membrane protein biogenesis are now quite well-understood. The importance of positively charged residues is firmly established, although their effects can vary depending on, *e.g.* the membrane potential and the lipid composition of the membrane (16). There are still aspects of the molecular mechanism responsible for the translocon-mediated insertion of transmembrane helices (TMHs) into the membrane that are not completely clear (17, 18); as an example, we do not know whether the TMHs first move into the center of the translocon channel and then exit through the so-called lateral gate in the translocon, or whether they slide along the lateral gate as they enter the membrane (Fig. 6). A question of mostly academic interest, for sure, but of the kind that membrane protein nerds like to mull over.

An interesting twist on the topology problem emerged around 2005, some years after the move to Stockholm University. We had started working with David Drew, then a Ph.D. student in Jan-Willem de Gier's lab in the department, who spearheaded a project on the use of GFP fusions to optimize the conditions for membrane protein overexpression and purification (19, 20), and we also decided to use GFP fusions for a proteome-wide topology mapping of *Escherichia coli* membrane proteins. It was already known that GFP becomes fluo-

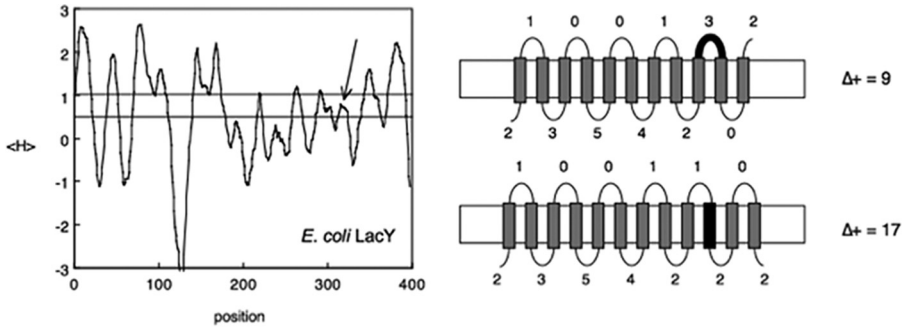

Figure 4. The TOPPRED predictor applied to the *E. coli* inner membrane protein LacY (13). Segments of intermediate hydrophobicity, falling between the two lines on the hydrophobicity plot, are considered "putative" transmembrane helices (*arrow*, *left panel*; *black*, *right panel*). All segments of high hydrophobicity, falling above the *upper line*, are considered "certain" transmembrane helices (*gray*, *right panel*). All topology models where each putative transmembrane helix is either included or excluded are constructed, and the one with highest positive charge bias is chosen as the most likely topology (*bottom model*, *right panel*; the number of Arg+Lys residue in each loop is shown). Reprinted from Journal of Molecular Biology. von Heijne, G. Membrane protein structure prediction hydrophobicity analysis and the positive-inside rule. *J. Mol. Biol.* 1992; 225:487–494. © Elsevier (13).

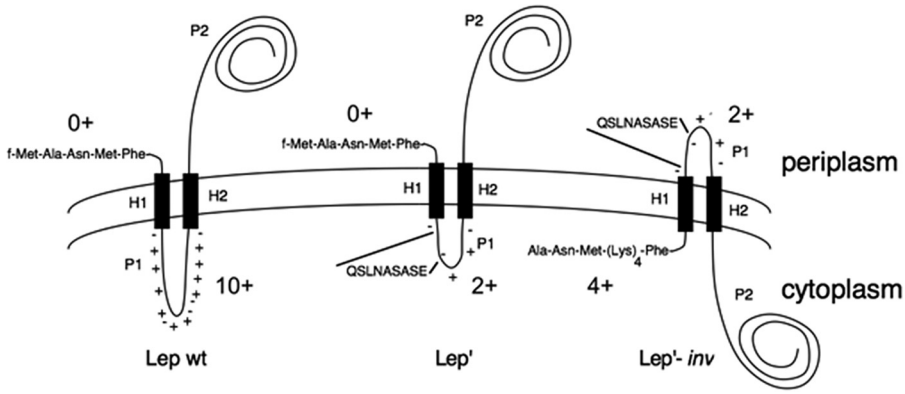

Figure 5. Turning Lep on its head. Deleting most of the positively charged P1 loop (*Lep'*) and adding four positively charged lysines to the N terminus (*Lep'-inv*) was sufficient to make the protein insert into the inner membrane in an inverted orientation (15). Reprinted from Nature. von Heijne, G. Control of topology and mode of assembly of a polytopic membrane protein by positively charged residues. *Nature*. 1989; 341:456–458. © Macmillan Publishers Limited, part of Springer Nature (15).

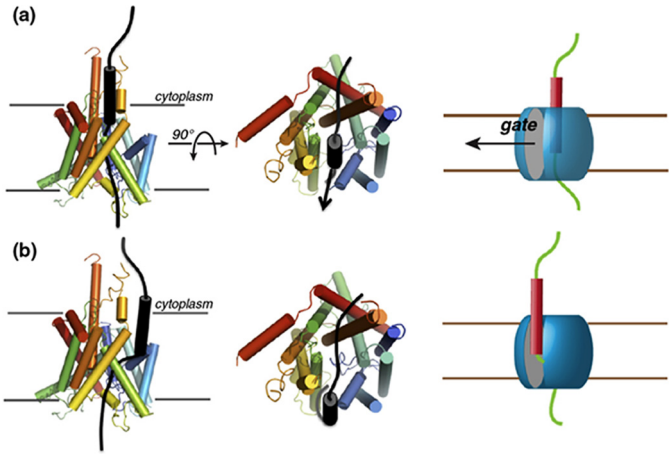

Figure 6. Two models for translocon-mediated insertion of a TMH. *a*, the "In-out" model. The TMH (in *black*) first moves all the way into the central translocon channel (the *E. coli* SecYEG translocon is shown), and then exits through the lateral gate. *b*, the "sliding" model. The TMH slides along the outer part of the lateral gate into the membrane. The leading polar segment penetrates through the lateral gate and is shielded from lipid contact. Reprinted from Journal of Molecular Biology. Cymer, F., von Heijne, G., and White, S. H. Mechanisms of integral membrane protein insertion and folding. *J. Mol. Biol.* 2015; 427:999–1022. © Elsevier (18).

rescent only in the cytoplasm and not in the periplasm, and from work in Jon Beckwith's lab, that alkaline phosphatase (PhoA) has the opposite behavior, *i.e.* it is active in the periplasm but not in the cytoplasm. So, inspired by the then emerging high-throughput biology craze, we decided to make C-terminal GFP and PhoA fusions to all the inner membrane proteins of *E. coli*, determine the cytoplasmic/periplasmic location of the C terminus based on the relative GFP and PhoA activities, and finally constrain a topology-prediction algorithm by the experimentally determined location to increase the reliability of the prediction. Amazingly, this worked, and a bunch of people in the lab (Dan Daley, Mikaela Rapp, Karin Melén, Erik Granseth, David Drew) obtained data for some 600 proteins out of the 700 that we started with (21). But the most interesting result was an unexpected one: six proteins scored high on both GFP and PhoA activity, suggesting that they might have dual topology, *i.e.* they might insert into the membrane in a ~50–50 mix between C_{in} and C_{out} orientations. Further experiments showed that this was in fact the case for all six proteins, and led to a number of follow-up studies where we mapped out the sequence determinants leading to dual topology—mainly the distribution of positively charged residues in the protein, as might have been expected (22–24). Evidently, there is something to be said for high-throughput studies!

Beyond reading papers and embarking on high-throughput fishing expeditions, another great way to increase the probability of productive chance encounters is of course to go to scientific meetings (and, as an added bonus, if you find yourself

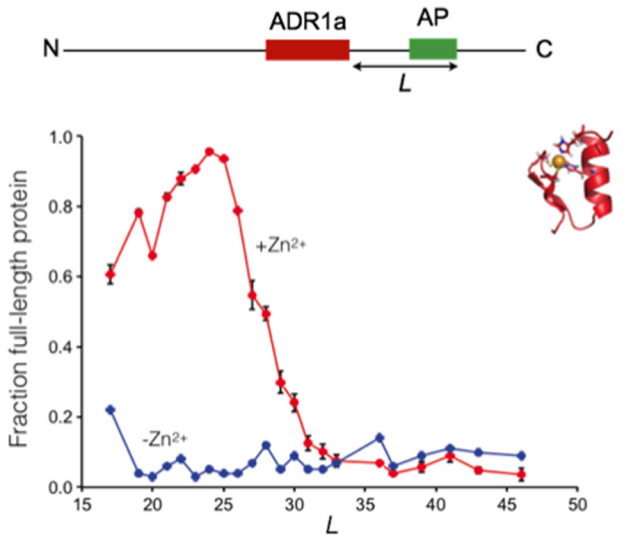

Figure 7. The zinc-finger domain ADR1a folds inside the ribosome exit tunnel. The construct shown at the top was translated *in vitro* in the absence (*blue curve*) and presence (*red curve*) of $ZnCl_2$. In the presence of Zn^{2+}, the cotranslational folding of ADR1a at $L \approx 22–27$ residues tether length generates sufficient force on the AP to prevent stalling, increasing the yield of full-length protein. At these tether lengths, ADR1a is still located well within the exit tunnel (35). Reprinted from Cell Reports. Nilsson, O. B., Hedman, R., Marino, J., Wickles, S., Bischoff, L., Johansson, M., Müller-Lucks, A., Trovato, F., Puglisi, J. D., O'Brien, E. P., Beckmann, R., and von Heijne, G. Cotranslational protein folding inside the ribosome exit tunnel. *Cell Rep.* 2015; 12:1533–1540. © Elsevier (35).

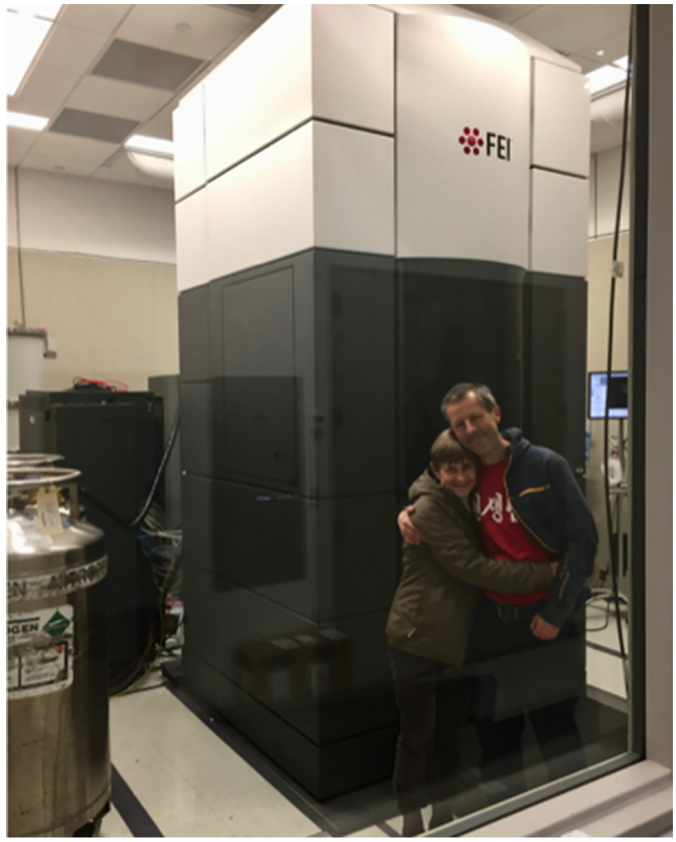

Figure 8. With my wife Anna in the cryo-EM lab at Rockefeller University (courtesy of Mark Ebrahim).

attending talks you don't enjoy, you get golden opportunities to think about your own science in peace). My best example: in May 2002, I found myself walking back to a hotel in Urbana–Champaign, chit-chatting with Steve White about transmembrane helices. He mentioned that he was keen to test a new biophysical hydrophobicity scale (25) *in vivo*, and it occurred to me that we already had the system to do this (26). This was the birth of the "biological hydrophobicity scale," worked on mainly by my Ph.D. student Tara Hessa, that kept both our labs busy for quite some time (27, 28). Because the biological hydrophobicity scale is based on measurements of translocon-mediated membrane insertion of model transmembrane helices, it directly reflects the full complexity of the biological system. There are some intriguing differences between the biological hydrophobicity scale and scales derived from measurements on peptides in membrane mimetics that still are not fully understood, and that may hold clues to the inner workings of the translocon (29).

And right now our lab is living yet another lucky break, which came about because I happened to read a paper where Koreaki Ito and his Ph.D. student Hitoshi Nakatogawa reported the discovery of the SecM translational arrest peptide (AP) (30). This paper showed that, once synthesized, the SecM AP "glues" itself into the ribosome exit tunnel and causes ribosome stalling (or "arrest," thus the name), serving as a control element to sense the activity of the co-transcribed SecA ATPase that helps move secretory proteins across the bacterial inner membrane. But what really made us jump was a subsequent paper from Don Oliver's lab (31), where he and his colleagues speculated that the SecM AP might be pulled loose from the exit tunnel by the SecA ATPase exerting force on the nascent chain, thereby lifting the translational stall. The next step was obvious: if this were indeed true, maybe we could use the AP as a transplantable force sensor to study various cotranslational processes that had hitherto been hard to analyze. Nurzian Ismail and Rickard Hedman, together with Nina Schiller and Martin Lindén, gave it a shot. It worked (32, 33). And we now study cotranslational folding of both soluble and membrane proteins using APs as force sensors (34–36) (Fig. 7). This, together with recent methodological breakthroughs in other labs (37–39), has breathed new life into the age-old question of whether and how cotranslational folding is different from *in vitro* folding of purified proteins, and exciting new results are coming out at a rapid rate.

Which brings me to my next-to-last point: electron cryomicroscopy (cryo-EM). The scene: a meeting in Nagoya in early 2013, ostensibly to celebrate the opening of a new EM center at Nagoya University but, in reality, to celebrate Yoshi Fujiyoshi's 65th birthday. The first two days are a little downbeat: people talk about 2D crystallography to determine structures of membrane proteins, but it is evident that this technique, important as it has been, will never become mainstream. Then, on the third day, something happens: Yifan Cheng from University of California, San Francisco (UCSF) shows a couple of breathtaking single-particle images of an ion channel protein recorded with a brand-new line of direct electron detectors. Total change of atmosphere: the old dream of getting atomic resolution structures by single-particle cryo-EM appears about to come true! The meeting ends in a mood of great excitement.

I leave Nagoya, not thinking too much about what I've just witnessed—after all, I don't think I had ever seen an electron

microscope in real life at that point. But the issue returns that fall, when, as I have been doing every year for almost 10 years, I go to spend a mini-sabbatical with Rod MacKinnon at the Rockefeller University in New York. Rod is busy trying to convince Rockefeller to invest in cryo-EM, and it slowly dawns on me that maybe Sweden should, too. I mention this when I get back home, and before I know it, I'm coordinating an application to the Knut and Alice Wallenberg Foundation and the Erling-Persson Family Foundation (of the H&M clothing stores) for a cryo-EM facility at the new Science for Life Laboratory in Stockholm. The application is funded, and in the summer of 2016, we have a working facility, ready to collect data. Plus a great group of newly recruited young scientists—Alexey Amunts, Marta Carroni, Julian Conrad, José Miguel de la Rosa-Trévin—to run the show. Even if it's not my own line of research, it's been quite an experience to help bring this new technology to Sweden. And, of course, we couldn't resist the temptation to integrate cryo-EM in some of our own projects: we now use APs to immobilize newly folded protein domains in the ribosome exit tunnel, and solve the structures using cryo-EM (35, 36) (Fig. 8). A little bit of structural biology is good for the soul!

Finally, let me say something about the Nobel Prize, since I've had the good fortune to be a member of the Nobel Committee for Chemistry for a rather long time. Not that it's possible to say much, since all our deliberations remain strictly classified for 50 years. But I can at least say this: as committees go, this committee is like no other. It holds more than 10 full-day meetings per year, no member is EVER absent from a meeting (the only time I've seen this happen was when a member died the night before one of our meetings . . .), and every member sees it as their most important job, period. And, believe it or not, I know of no other committee where there is so much laughter and good humor—it's the only way to cope . . .

So, my story ends with one of life's little ironies: tears over a Lucia costume turning into tails at a Nobel banquet. Except that you probably expect me to finish off with some wise morals distilled from 40+ years in science. Here they are:

- Life is ruled by chance.
- Read, read, read (even if it's in in French).
- When you get a break, stick with it. Breaks are rare.
- Collaboration is more fun than competition.
- Spend time in New York.
- Don't make your papers longer than necessary. Remember Samuel Beckett: "Every word is an unnecessary stain on silence and nothingness." Maybe I should just have stayed quiet . . .

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