Chaperonin studies: faith, luck, and a little help from our friends

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ABSTRACT **Basic cellular research is a trail. One follows one's nose toward what might be new understanding. When that leads to a need to employ unfamiliar or novel technology, it's both exciting and very worthwhile to form collaborations. Our early studies of chaperonins support such a philosophy, as detailed in the two stories that follow, written in deep appreciation of recognition by the E.B. Wilson Medal of the American Society for Cell Biology.**

DISCOVERY OF FOLDING FUNCTION

In Fall 1987, my fledgling laboratory—three graduate students, a technician, and me—set out to identify mutants of baker's yeast that affected the import of cytosolically synthesized precursors into mitochondria. Heeding the enjoinders of Gottfried Schatz and Walter Neupert, two giants of that field, that cells could not grow without producing new mitochondria (e.g., Baker and Schatz, 1991), we decided to produce our own temperature-sensitive conditional library, with the idea that import mutants would not be able to grow at a nonpermissive temperature. We mutagenized a strain of yeast programmed to express a Gal-inducible mitochondrial reporter protein, human mitochondrial ornithine transcarbamylase (OTC), which we would turn on by switching from glucose to galactose medium after shifting each mutant to the nonpermissive temperature. We would look to see whether our reporter protein could achieve enzyme activity as an indication that it could enter mitochondria, that its targeting peptide could be cleaved, and that it could assemble into an active homotrimer (Cheng *et al.*, 1987). We were seeking to uncover mutants affecting recognition and translocation into the organelles, as well as proteolytic maturation. Sure enough, activity-deficient/cleavage-defective mutants were identified, affecting what was shown to be the mitochondrial protease (Pollock *et al.*, 1988).

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But one night it dawned on us that a very interesting mutant might also be present in our library, one affecting the folding of newly imported proteins. After all, Gottfried Schatz had reported that proteins have to be unfolded to cross the mitochondrial membranes (Eilers and Schatz, 1986). There were also reports from Blobel (Chirico *et al.*, 1988) and Craig and Schekman (Deshaies *et al.*, 1988) indicating that proteins of the endoplasmic reticulum (ER) and mitochondria are maintained in such unfolded states in the cytosol by Hsp70 chaperones. What if there was a "chaperone" protein on the other side of the mitochondrial membranes that acted on the newly imported proteins to support their de novo folding to the native form? That sounded like a challenge to the studies of Christian Anfinsen, who had determined that polypeptides contain sufficient information in their amino acid sequences to reach native form (Anfinsen, 1973), and indeed all the import models of the time drew imported proteins as spontaneously folding/assembling to native form. It seemed worthwhile to look for a folding-defective mutant where the imported protein would reach a proteolytically matured form inside the organelles but be enzymatically inactive, occupying a misfolded state.

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Abbreviations used: ER, endoplasmic reticulum; OTC, ornithine transcarbamylase.

Remarkably, within several days, Ming Cheng, a young physician from Taiwan who had decided to shift direction to graduate training in Yale Genetics, came in with an autoradiogram showing mature-sized OTC subunits from inside an activity-dead strain. Using an affinity column that specifically bound assembled OTC (Kalousek *et al.*, 1984), our next door neighbor, Franta Kalousek, showed that the OTC subunits in this mutant simply passed through the affinity column without capture. We had to presume that the imported subunits were matured but then misfolded, unable to assemble. We decided to test a yeast endogenous mitochondrial protein for which we had antibody, the beta subunit of the F_1 ATPase. We observed the same result–at nonpermissive temperature the newly imported subunits reached mature size but failed to incorporate into the ATPase stalk structure. It seemed unreal that this mutant could be affecting the de novo folding of newly imported proteins. No one would ever believe this result from our tiny group. Now Spring 1988, the phone rang, and it was Walter Neupert and Ulrich Hartl from Munich. They had heard that we were studying yeast mutants affecting import and wondered whether we could use a little biochemical help in analyzing them. WOW!! COULD WE EVER! So I went over to Munich the next week and presented the mutants, saving the putative folding mutant for last. That really got their attention. They worried that maybe the importing proteins were trapped in the import channel, able to have their signal peptides cleaved but unable to fold because of their "stuck" topology. I tried to allay that worry by indicating that the species we saw in our organelle preparation were abundant, potentially reflecting that they were not being proteolytically "bitten"—but the obvious experiment to do was an in vitro import assay with isolated mutant mitochondria and confirm that proteins were going all the way into the matrix by resistance to added protease. I sent Ulrich the strain, and he called back excitedly two weeks later to indicate that, yes, the proteins were going in and that they must then be misfolding.

Now the expertise of a major import lab could be brought to bear on this seemingly heretical mutant yeast strain. Many additional endogenous yeast proteins were examined with antibodies available in the Munich lab. One of the most exciting results involved the Rieske iron-sulfur protein, a monomer during its lifetime in the matrix space, where it undergoes two signal peptide cleavages before being inserted into the inner membrane (Hartl *et al.*, 1986)—in the mutant, there was either no cleavage or a single event. This argued that it was the folding of a monomer and not the assembly of already folded proteins that was affected. But what was the gene itself that was affected in the mutant strain on which we were focused?

Ming Cheng transformed a yeast plasmid library into the mutant strain and plated at 37°C and then sequenced the rescuing gene. An open reading frame for an ~58 kDa protein was observed in all of the rescuing plasmids. That prompted us to make a phone call to Richard Hallberg in Iowa, who had described a 60 kDa heat inducible protein in *Tetrahymena thermophila* mitochondria in 1987, which formed a ring assembly (McMullin and Hallberg, 1987). We imagined that we might be studying the yeast homologue. So on a Saturday afternoon in August 1988, he and Ming Cheng sat on the phone and matched base for base the sequence of our rescuing gene with the yeast homologue of his gene isolated by antibody screening of a lambda gt11 library. So that was it—this connected folding in mitochondria to a double ring assembly, a chaperonin as John Ellis had dubbed them. On that same afternoon, by chance, I was picking Ulrich up at JFK, the two of us heading to a Gordon Conference the next day. We returned to my tiny beach house, and there was the lab group standing on its dock, jumping up and down with the sequencing autoradiograms in hand demonstrating the dead matchup. And so began a 20-year exploration into how these kinds of double ring machines mediate protein folding. (See Cheng *et al.*, 1989, for the report of the folding-defective mutant; Reading *et al.*, 1989, for Hallberg's sequence; and Hemmingsen *et al.*, 1988, for the relatedness of chaperonins of bacteria and chloroplasts.)

SEEING THE MACHINE BY X-RAY CRYSTALLOGRAPHY

In 1991, an administrative mission took me across campus to visit Paul Sigler, a crystallographer newly arrived from the University of Chicago, resulting in an immediate discussion about the chaperonin double rings, their symmetries, and the potential of seeing them at high resolution by crystallography. Paul was convinced that we would never figure out the mechanism without a structure. In that moment, a 10-year collaboration that was like a father–son relationship formed. Paul and I shared Chicago roots, the Bulls, the Bears, a love of jazz, and much else. He taught us the tenets of crystallography, and we set out to generate well-diffracting crystals. It was a three-year project with nothing in hand until the very end, a matter of major faith. We initially tried thermophilic versions of the bacterial chaperonin, GroEL, but we never obtained any crystals that diffracted beyond 12 Å. Then, what I believe to be a key event occurred. The Sigler lab moved from its uptown location down into the Boyer Center right next door to us. This catalyzed a daily ongoing discussion between all of us on the problem of expressing and crystallizing GroEL. Andrzej Joachimiak and I took up the former challenge and soon found that a *trc*-driven expression plasmid produced *Escherichia coli* GroEL from its operon as virtually the only protein upon induction. That made purification a single-step affair, and trays could be set up with that material. Thus a large range of molecules could be tested, necessary because wild-type *E. coli* GroEL did not behave well in the various trials we or others had set up. David Boisvert, with excellent crystallization experience from a period in the Steitz lab, taught Kerstin Braig and me how to set up trays and vary conditions systematically (there were no robots at the time). The three of us set up a myriad of crystallizations of different variants of GroEL. After a year, Kerstin pulled up a beautiful orthorhombic crystal that diffracted on the area detector next door to 2.4 Å. Paul came running into the room and began dancing about as he looked at the diffraction pattern. Needless to say, we went to the Cornell synchrotron that weekend and collected, with the help of his group, a 2.8 Å native data set. It was a 24-hour fire drill to collect this gigantic molecule (840 kDa) on image plates, six plates at a time. Immediately following, David's recently produced monoclinic crystal was collected, diffracting to better than 2.0 Å. The molecule that behaved in both crystal forms turned out to be a PCR-directed variant of GroEL with two relatively benign substitutions in its ATP binding domain—we had been calling it wild type!

The next week, Paul sat us down and delivered a four-hour lecture on how to prepare heavy atom derivatives for phase determination. Kerstin promptly produced an ethyl mercury derivative that was isomorphous, and now Zbyszek Otwinowski, a senior member of Paul's group, took over and solved the structure in a single day. He had been thinking about how to carry out sequential multiparameter searches to identify the heavy atom positions. Three sites per subunit were found (corresponding to three cysteines per subunit). So on a Saturday morning in Fall 1993, Paul, Zbyszek, and I stood in front of a screen in a darkened room and there was the GroEL molecule in front of us at 10 Å resolution, soon to be 2.8 Å when Zbyszek applied sevenfold noncrystallographic symmetry averaging/phase extension. It was like looking at a sacred object. A

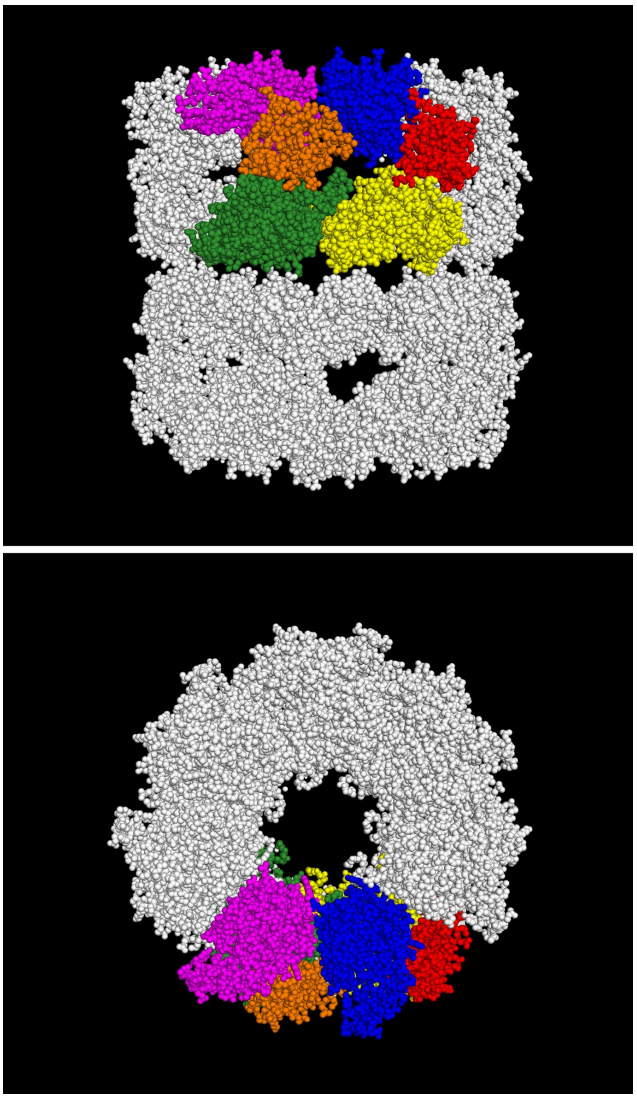

FIGURE 1: Space-filling model of the refined unliganded GroEL molecule initially crystallized, showing the back-to-back seven-membered rings (PDB 1OEL; Braig *et al.*, 1995). Upper panel, side view; lower panel, end view looking down the sevenfold symmetry axis. The molecule is 135 Å in diameter and 145 Å in height and contains a central cavity in each ring that is ~45 Å in diameter. The two central cavities are separated by disordered C-terminal tails of the GroEL subunits (not visible crystallographically but visible as masses in EM) that project into and block the cavities at the equatorial levels. Two neighboring subunits from the top ring are colored for their domain structure. At the waistline, green and yellow domains are *equatorial* domains, which make tight contacts both around a ring and between rings and comprise the base of the assembly. Each equatorial domain houses an ATP binding pocket at its inside aspect. Orange and red *intermediate* domains with hinges at their top and bottom aspects mediate covalent connection of the equatorial to the terminal *apical* domain of each subunit. Two apical domains are colored here in purple and blue. The apical domains contain at their cavity-facing aspect hydrophobic surfaces that multivalently capture nonnative polypeptides exposing hydrophobic surface that will be buried to the interior in the native state. The hinged intermediate domains open in the presence of ATP binding, causing elevation and rotation of the apical domains, dislocating the hydrophobic cavity surface, allowing binding of the cochaperonin "lid" GroES, and releasing polypeptide into a now encapsulated hydrophilic cavity where productive folding occurs in isolation without the possibility of aggregation.

dream had come true. What followed was refinement and presentation of the model of unliganded GroEL (Braig *et al.*, 1994; see Figure 1), structure/function studies (Fenton *et al.*, 1994; Weissman *et al.*, 1994, 1995), structure of GroEL with bound ATPγS (Boisvert *et al.*, 1996), structure with ADP and the cochaperonin GroES bound (Xu *et al.*, 1997), and mechanism studies (e.g., Weissman *et al.*, 1996; Rye *et al.*, 1997). Once again, faith, luck, and help from our friends had opened up a new avenue to seeing how this machine could assist de novo protein folding in the cell.

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