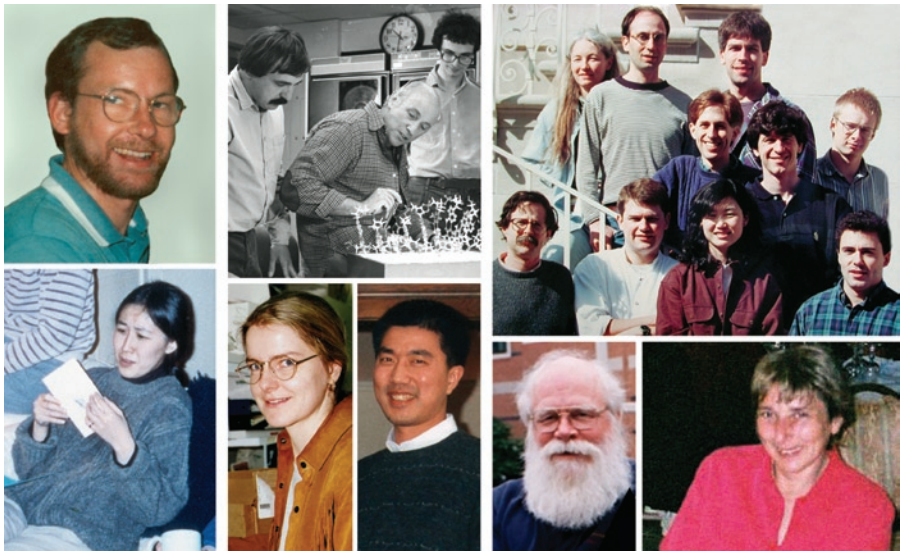
Protein folding in the cell: an inside story

Arthur L Horwich

The final step of information transfer from DNA to effector protein involves the folding of newly translated polypeptide chains into characteristic three-dimensional active structures. In the fall of 1972, while working in a biochemistry laboratory as an undergraduate at Brown University, I heard about an astonishing experiment for which Christian Anfinsen was receiving the Nobel Prize in Chemistry. Anfinsen and his co-workers had unfolded purified RNase A by reducing its disulfide bonds and exposing it to a denaturing agent, and then asked whether the protein could find its way back to the enzymatically active native state upon removal of the reducing agent and denaturant[1]. Amazingly, it did. I was utterly haunted by the beauty of this observation and by the profound conclusion that the primary structure of a protein contains all the information necessary to direct its folding to the native functional state. I could never have imagined that, many years later and in a completely unexpected fashion, I would have something to add to something so fundamentally beautiful.



Contributors to the discovery of chaperonin action and to understanding of the chaperonin mechanism. Top left: Ulrich Hartl, *c.* 1988; bottom left: Ming Cheng, 1988. Top center: Andrzej Joachimiak, Paul Sigler and Zbyszek Otwinowski, *c.* 1990; bottom center: Kerstin Braig, *c.* 1993, and Zhaohui Xu, *c.* 1996. Top right: the 1996 team, members of which dissected the topology and reaction cycle. Shown left to right in top row, Krystyna Furtak, Jonathan Weissman, George Farr; in middle row, Matt Goldberg, Hays Rye, Oleg Kovalenko; in bottom row, myself, Steve Burston, Suwon Kim, David Boisvert. Bottom right: Wayne Fenton, timeless, and Helen Saibil, *c.* 2003.

I finished my undergraduate and medical training at Brown and went to Yale for pediatric residency, but, by the second year of training, I was missing the laboratory. I became captivated by the problem of malignant transformation mediated by single viral genes, and, by the end of my residency, I had amassed on my nightstand a stack of several hundred papers on the topic.

I then moved to the Salk Institute to work with two masters of the field, Walter Eckhart and Tony Hunter, who taught me molecular biology and biochemistry. I watched Tony discover tyrosine phosphorylation, a fortuitous finding that resulted from his using an aged electrophoresis buffer for separating phosphoamino acids (but as Pasteur noted, "...chance favors the prepared mind"). Observing Tony carry out his own experiments day by day as a role model was already quite an experience, but it went further. He took the time to teach me how to think about a problem, he taught me good technique directly and his tutelage solidified my love of science and of being at the bench.

By the end of my 3 years there, I wanted to add a clinical element to my research—particularly because it was possible by then to isolate mammalian sequences that might have to do with disease. I therefore returned to the Yale School of Medicine to learn clinical genetics and to work with Leon Rosenberg on the urea-cycle enzyme ornithine transcarbamylase (OTC), the absence of which causes X-linked inherited lethal ammonia intoxication. We painstakingly isolated, cloned and sequenced cDNA for the nuclear-encoded precursor of this homotrimeric mitochondrial enzyme. Our efforts were rewarded with seeing a mitochondrial targeting peptide sequence and showing that it alone was sufficient to direct proteins into mitochondria[2]. Moreover, these experiments also made possible DNA-based diagnosis of OTC deficiency.

I was offered the chance to move across the hall from Leon's lab as a junior faculty member—a dream come true—and decided that my first project would be to test whether the human OTC precursor, when expressed in genetically manipulable yeast, could enter the mitochondria and reach active form. Happily, it could, reflecting the conserved nature of the import pathway[3].

Thus, our fledgling group of four (Krystyna Furtak, Ming Cheng, Bob Pollock and me) decided to carry out a genetic screen in yeast for mutants affecting import of our OTC reporter into the organelles, as, presumably, there were receptors, channels and processing enzymes responsible for the process. We screened a bank of yeast with temperature-sensitive lethal mutations, expecting that

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blocked import would be lethal to the cell. We programmed the human OTC precursor to be turned on after a temperature shift and then asked about its fate. Was any OTC enzyme activity produced? Was the targeting peptide removed? Indeed, early on, we isolated mutants in which the cleavage of the targeting peptide was affected.

But then, one night in the fall of 1987, after a long day of replicating hundreds of plates and carrying out western blots (what we referred to as "doing the laundry"), my student Ming Cheng and I looked across our benches at each other and wondered, could there be such a thing as a folding machine in the mitochondria that is required to refold the imported proteins? Our question rested on several prior observations. For example, under heat-shock conditions, Hugh Pelham had shown that a 70-kDa heat shock–inducible protein (Hsp70) could act as a "molecular crowbar" to reverse protein aggregation[4]. James Rothman's group had shown that Hsp70 could mediate the disassembly of clathrin coats[5]. But most relevant to our mitochondrial system was the observation from Gottfried Schatz's group that proteins have to be unfolded in order to cross the mitochondrial membranes[6]. If this was the case, what happened on the other side of the mitochondrial membranes? Did proteins simply refold and assemble spontaneously, as in Anfinsen's experiments? Or was there an active 'machine' that folded the newly entered proteins to their native form (**Fig. 1a**)? The latter seemed almost heretical to consider, but we knew what the phenotype would look like: the OTC precursor would enter the matrix space and its signal peptide would likely be cleaved, but there would be no enzymatic activity. Within a week of our first having the thought, there it was—a mutant we called *mif4* (mitochondrial import function 4) that had exactly those features.

We began to analyze the mutant by looking at an endogenous yeast mitochondrial protein, the β-subunit of the F_1ATPase, to assess whether the subunit had been assembled into the ATPase complex (**Fig. 1b**). In *mif4*, it had not. We had a hard time believing that we had found what we had originally speculated might exist.

Early in 1988, the phone rang, and Walter Neupert and Ulrich Hartl told us that they had heard we had isolated a number of mitochondrial import mutants, and they wondered whether we would like any assistance with the biochemical analysis. It took me less than a second to respond that I would love nothing better than to collaborate with such an able and distinguished group. I went to Munich the following week and presented the *mif4* story. Walter and Ulrich were a little worried that maybe we

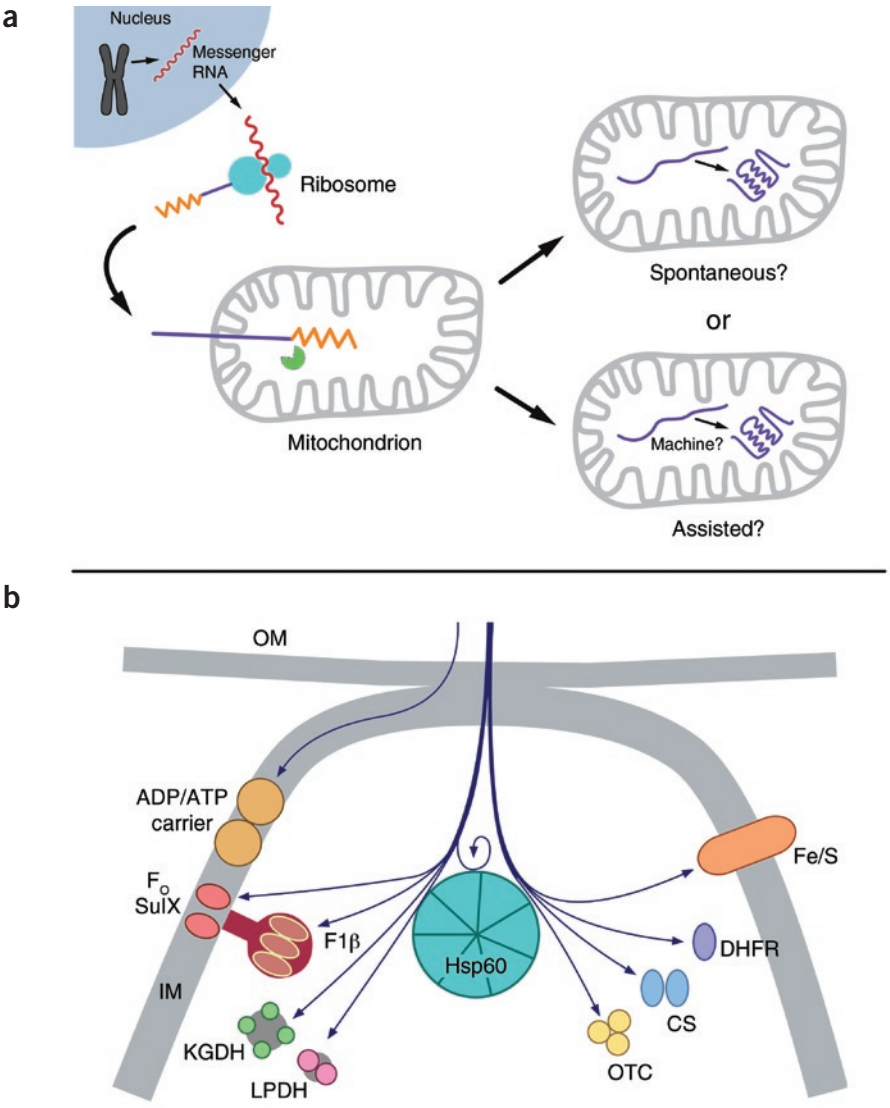

Figure 1 Mitochondrial protein import and the demonstration that a folding machine assists refolding of newly-imported proteins. (**a**) Most mitochondrial proteins are encoded in the nucleus, translated in the cytosol and targeted by N-terminal signal peptides (orange). It had been shown that, in order to cross the mitochondrial membranes, proteins had to occupy an unfolded conformation[6]. During and after translocation across the membranes, the signal peptide is cleaved by a protease inside the organelle (green 'PacMan'). The question we asked concerned refolding of the imported mature-sized proteins to the native state. Did they spontaneously refold, as models at that time suggested (top), or were they assisted by a putative molecular machine (bottom)? (**b**) Proteins that matured to their native, active form in the mitochondrial matrix compartment were affected by the *mif4* mutation in the identified molecular machine, called Hsp60 (heat-shock protein 60). The studies of Rieske iron-sulfur protein (Fe/S) and dihydrofolate reductase showed a monomeric polypeptide folding step was involved, as opposed to oligomeric assembly of already-folded subunits. Studies of α-ketoglutarate dehydrogenase (KGDH) and dihydrolipoamide dehydrogenase (LPDH) were reported in ref. 35. Figure adapted from ref. 36.

weren't seeing fully imported proteins in our mutant; that is, that the reason the proteins weren't active was that they were jammed in the import channel.

To rule out this possibility, they offered to carry out protease protection assays with mitochondria from the *mif4* mutant. Two weeks later, Ulrich called in a state of elation to say that, yes, proteins were going fully into the organelles, to the protease-protected interior, but were not reaching native form. He then carried out an experiment that assured me we were studying a folding defect. The Rieske iron-sulfur protein (Rieske Fe/S) is an inner-membrane protein that is first imported to the mitochondrial matrix and then cleaved twice while a monomer before being inserted into the inner membrane. In *mif4* mitochondria, Rieske Fe/S was either

uncleaved or cleaved only once. It apparently could not achieve the correct conformation for completion of cleavage and membrane insertion[7]. Lest there be any remaining doubt, Ulrich and Joachim Ostermann subsequently carried out a set of compelling experiments importing a mitochondrial targeting peptide–attached monomeric dihydrofolate reductase, showing its folding to require the *mif4*-encoded action[8].

It was high time to find out what gene was affected in the *mif4* mutant. The answer came on a Saturday afternoon in the summer of 1988. Ming Cheng had rescued the *mif4* mutant with a yeast library, and one gene had come up multiple times. After sequencing the gene, she thought to call up Richard Hallberg in Iowa, who had been working on a mitochondrial heat-shock protein in *Tetrahymena thermophila* that appeared to be arranged in a ring assembly[9] and who, we knew, was sequencing the homologous yeast gene. Meanwhile, my son and I had gone down to New York to pick up Ulrich at the airport, as the two of us were going to a Gordon Conference the next day. We got back to our little house on the beach to find Ming and other members of the lab literally jumping up and down. She and Hallberg had compared gene sequences and found that they were a dead match! The folding activity was coming from a ring machine, which we, together with Hallberg, dubbed heat-shock protein 60 (Hsp60) because the subunit was ~60 kDa[7].

Despite the name, the function of Hsp60 was not confined to heat-shock conditions. When the gene encoding Hsp60 was deleted, yeast were dead at all temperatures[7,10]—its folding function was essential under all conditions (**Fig. 1b**). We presumed that we must be studying a function that assists protein folding by preventing proteins from misfolding under cellular conditions—that is, 37 °C and high protein concentrations. In other words, Anfinsen's principle—that primary structure guides folding—was operative, but mistakes could happen under the conditions found in a cell. This had been the case when mammalian proteins were expressed in bacteria—the proteins often misfolded and formed aggregates. Likewise, a number of human proteins carrying disease-causing mutations were known to aggregate. Consistently, in *mif4* cells lacking Hsp60 function, many imported proteins now lodged in insoluble aggregates[11]. Thus, the action of the ring machine was to somehow provide kinetic assistance to the process of protein folding.

Inspection of the Hsp60 amino acid sequence made clear right away that the protein was related (with ~60% identity) to the subunit of the bacterial ring assembly GroEL (which is composed of two back-to-back, seven-subunit rings) and also to a chloroplast protein, the Rubisco subunit binding protein (also a ring assembly). The GroEL and Rubisco macromolecular complexes had been implicated, respectively, in the oligomeric assembly of phage particles (by Costa Georgopoulos[12] and others[13]) and in the assembly of the abundant CO_2-fixing enzyme Rubisco (by John Ellis[14]). In fact, GroEL and the Rubisco binding protein had recently been shown to have closely related amino acid sequences[15]. But now it seemed more likely that the role of these ring machines was to mediate polypeptide chain folding.

If GroEL mediated protein folding in the bacterial cytosol, could there be an analog in the eukaryotic cytosol? The answer soon came from Jonathan Trent, who worked with my group and Ulrich's on a thermophilic archaebacterial heat-shock protein of 60 kDa. Jonathan showed that, when this protein was highly induced, it conferred thermotolerance to *Sulfolobus shibatae*. In the electron-microscopy analysis of the purified protein, which behaved as a megadalton complex, we were stunned to observe a stacked double-ring assembly, with nine subunits per ring. But most revealing was the protein's predicted primary structure[16], which included regions at either end that were related to GroEL, Hsp60 and Rubisco binding protein—which we now know to be the ATPase domain of these machines—as well as a middle region that bore essentially no relation to those assemblies but that was closely related to the eukaryotic t-complex polypeptide-1, a cytosolic protein that had been implicated in tubulin biogenesis[17]. In short order, we found that this protein was a subunit of a double-ring assembly, now known as TRiC (TCP1 ring complex) or CCT (complex containing TCP1), that has eight distinct subunits per ring[18] and is required for proper folding of tubulin[19], actin[20] and a host of other proteins.

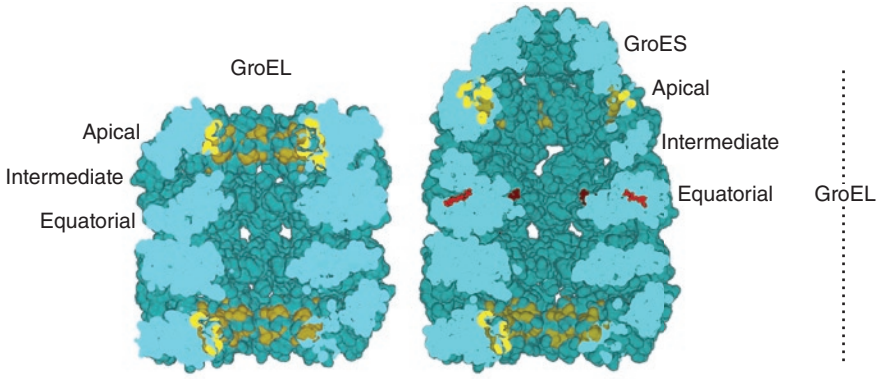

Figure 2 Cutaway views of crystallographic models of unliganded GroEL and an asymmetric GroEL-GroES-ADP$_7$ complex. (**a**) The model of GroEL immediately demonstrated two back-to-back rings with three domains in each of the seven subunits per ring: an apical domain, the collective of which forms the terminal portions of the GroEL cylinder, with hydrophobic polypeptide binding sites (yellow) on the inside aspect; an intermediate hinge-like domain; and an equatorial domain, the collective of which forms the base of the assembly and houses a nucleotide binding site facing the cavity (see nucleotides colored red in **b**). (**b**) In the GroES-bound state, the bound GroEL ring has undergone large rigid-body movements that result in elevation of the apical domains to form contacts with GroES through the same hydrophobic surface that bound polypeptide. Thus, the hydrophobic binding surface is elevated and twisted away from the large, now GroES-encapsulated cavity, forming a cavity with a hydrophilic, electrostatic surface. (Note the absence of hydrophobic side chains exposed to the cavity in the upper, GroES-bound ring.) In the course of these movements, the polypeptide substrate initially bound is released into the large folding chamber, where it folds in solitary confinement without the chance of aggregation.

Structure and mechanism

At this point, the central question became: how do the chaperonin ring machines assist protein folding? Work of George Lorimer and his colleagues[21] and of Ulrich's group[22], both using the bacterial GroEL system, showed that a folding reaction could be reconstituted *in vitro* in two steps: (i) binding of non-native protein in a 1:1 stoichiometry with the ring assembly and (ii) release and folding, triggered by ATP and a ring complex of seven identical 10-kDa subunits called GroES. Soon afterward, both Ulrich, with Wolfgang Baumeister, and our group, with Joe Wall and Jim Hainfeld, showed by electron microscopy that the polypeptide was bound in the central cavity of an open ring[23,24]. But the folding reaction still seemed opaque, and there were as many models as investigators in the field.

In the spring of 1990, our experimental direction for the next 10 years was dictated by a routine administrative meeting with Paul Sigler across town on the main Yale campus. He was a newly arrived crystallographer from the University of Chicago, fresh from solving the structure of a *trp* repressor-operator com-

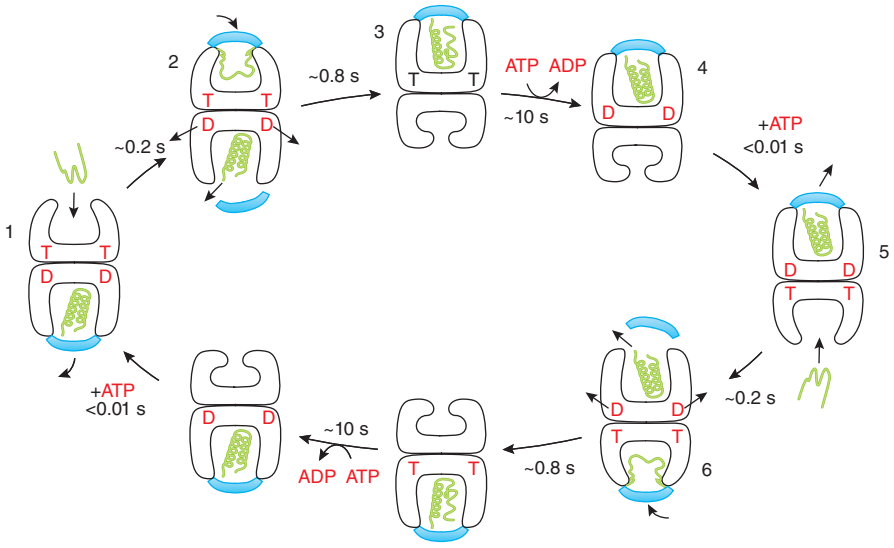

Figure 3 The GroEL-GroES reaction cycle. The cycle is directed via the binding and hydrolysis of ATP in respective GroEL rings (red T, ATP; red D, ADP). Horovitz and co-workers showed early that ATP binds cooperatively within a GroEL ring but anticooperatively between rings[37], such that when one ring has seven ATPs, the other does not bind ATP. Binding of GroES (blue 'lid') is dependent on the binding of ATP to the GroES-associated ring[38], resulting in the formation of asymmetric complexes, in which folding proceeds inside the GroES-bound GroEL ring. A cycle begins with polypeptide (green 'squiggle') binding to the ATP-bound ring of an asymmetric complex (1) and is immediately followed by GroES collision with that ring, producing a state (2) in which both polypeptide and GroES are bound to the apical domains (this prevents escape of the polypeptide). Once GroES docks, there are further large rigid-body movements that produce the domed folding chamber (3). Folding proceeds in this longest-lived of the GroEL states. After ~10 s, ATP hydrolysis occurs (4), weakening the assembly and permitting entry of ATP into the *trans* ring (5)[39]. This sends an allosteric signal that ejects the ligands from what had been the folding-active ring (6), such that GroES, polypeptide (whether folded or not) and ADP depart, while a new folding cycle commences on the newly ATP-bound ring[40]. The machine thus alternates back and forth, using one round of seven ATPs at a time to nucleate a folding-active ring and dispatch the previous one. (Figure adapted from ref. 41.)

plex. The administrative matter was solved in 30 seconds, and Paul turned immediately to our chaperonin work: the nature of these ring assemblies, their beautiful symmetries and the possibility of crystallizing them. Once again, within a second, we became collaborators, a relationship that ended only with Paul's untimely death in 2000.

Paul dragged Zbyszek Otwinowski into the room to consider the size and symmetry of the chaperonin rings. "Not manageable with current computing," Zbyszek told us, "but it'll take you years to crystallize it anyway and, by then, computing will be ready." He was correct. We tried thermophile versions of GroEL and monomeric versions of *Escherichia coli* GroEL, but never attained decent crystals. Meanwhile, Paul's group and mine had moved into side-by-side labs in the newly constructed Boyer Center, where our daily interaction accelerated to a wonderful pitch. Paul and his group were delighted to teach us crystallography. Paul became my scientific father—we shared Chicago (I had grown up there), the Bears football team, jazz, medical training and residency experience, and our love of science.

Andrzej Joachimiak, a member of Paul's team, and I succeeded in massively overproducing GroEL—so that it was the only band in an SDS gel when one cracked the cells. We could overproduce any variant, and immediately my student Kerstin Braig set up a massive wall of trays of various mutant molecules we designed. Astonishingly, 3 years into the project, a beautiful crystal form in ammonium sulfate appeared, produced from a PCR-derived variant that we later determined had accidentally acquired two functionally benign mutations. We tested the crystals on Paul's local X-ray machine, and they diffracted to a 3.4-Å resolution! Paul came running into the room, completely excited and delighted. Three days later, we went to collect native X-ray data at the Cornell synchrotron. GroEL was such a large molecule that it took us 24 hours of continuous data collection on image plates, a real fire drill, to recover the needed data; many members of the Sigler group stayed up all night to assist. Then, several months later, Kerstin obtained a heavy-atom derivative of GroEL that allowed Zbyszek to solve the structure in a single day (no mean feat with 42 heavy-atom sites per GroEL complex).

I was in the UK at the time, and casually called the lab to learn, to my spectacular delight, that they were looking at the structure of GroEL at 10-Å resolution. I got on the next plane home, and it was a climactic moment, that morning in the fall of 1993, when Zbyszek, Paul and I went into a darkened room and saw the model of the folding machine on the graphics display. Because of the structure's large size, we could only look at one ring at a time, and it was hard to move the model around on the display. But we could see that we were going to learn how this beautiful work of nature could function. After rounds of averaging to achieve higher resolution, Zbyszek traced the chain, and we could now see the GroEL domains: an equatorial domain, the collective forming the waistline of the cylinder, a stable base for the machine, with each subunit housing a pocket that we thought must be the ATP-binding site, and an apical domain, the collective forming a 45-Å-diameter cavity at each end of the cylinder where we thought non-native polypeptide must be bound[25] (**Fig. 2a**).

Mutation studies confirmed the ATP-binding site, and a co-crystal of GroEL-ATP, thanks to the efforts of David Boisvert in my group, working with Jimin Wang[26], showed the stereochemistry of binding. But our initial mutational tests for polypeptide binding sites were not successful. We then looked at one of Helen Saibil's electron microscopy studies and observed masses of density in the central cavity docked directly against the apical, cavity-facing surface[27]. We went back to the structure and immediately recognized the set of hydrophobic side chains exposed to the cavity on the face of the apical domain, which must be recognizing polypeptide (**Fig. 2a**). We came in one morning after mutagenizing each of these sites individually to find that every one of the mutants was dead; indeed, when the corresponding mutant GroELs were produced and purified, none could bind non-native polypeptide[28]. Thus, GroEL specifically recognizes non-native proteins by their exposed hydrophobic surfaces, which in the native protein are buried in the interior, and binds them with its own hydrophobic surface. In the absence of chaperonin binding *in vivo*, proteins exposing such surfaces are prone to aggregation, binding to each other through hydrophobic interactions. Thus, it was clear that one of the kinetic actions of GroEL was to forestall such aggregation by recognizing exposed hydrophobic surfaces and sequestering them.

But how did the folding part of the GroEL reaction work? What did ATP and GroES do? It was clear that GroES generally bound at one end of the GroEL cylinder at a time[29], and could only bind to a nucleotide-bound ring (**Fig. 2b**). But where did the non-native protein come in? Did it bind on the empty ring,

or could it occupy a position encapsulated underneath GroES? Helen Saibil's work suggested there was sufficient room underneath GroES to house a protein molecule[27]. Jonathan Weissman in my lab carried out a topology experiment, using order of addition (that is, either polypeptide then nucleotide-GroES or vice versa) and proteolysis, that confirmed that a non-native protein could indeed be protected from protease while bound underneath GroES. Then, Wayne Fenton made both so-called *cis* ternary complexes (in which the polypeptide bound to a GroES-associated ring) and *trans* complexes (in which the polypeptide bound to the ring opposite GroES). He used a single-round experiment to ask which would be productive. The lab was evenly divided on what the outcome might be. The answer was definitive: *cis* complexes were productive, yielding a properly folded polypeptide, whereas *trans* complexes were not[30].

In a further test, a long-lived GroEL-GroES complex was made using a single-ring version of GroEL. The monomeric substrate protein rhodanese went all the way to its native active form inside the *cis* complex once ATP and GroES were added[31], and the rate of recovery of its activity was the same as in a cycling reaction, where we had observed that the polypeptide must leave with each turnover of a ring of ATP whether it is folded or not[32]. Thus, under normal conditions, if the polypeptide has reached native form during folding in the *cis* ring, it goes out into the cell and carries out its function (**Fig. 3**). If a released polypeptide has not reached native form, its exposed hydrophobic surfaces promote its rapid return to GroEL and another trial at refolding[32]. For any given round of folding, we observed that only a fraction of molecules reached native form. Because no intermediates could be detected, the process appeared to be an all-or-none one. But what did a *cis* cavity look like, and how was it produced?

The answer to this question became evident both from electron microscopy studies by Helen Saibil[33] and from a GroEL-GroES crystal structure piloted by Zhaohui Xu from Paul's lab[34]. The apical domains underwent a large (60°) rigid-body elevation and an even larger (100°) clockwise twist in the presence of adenine nucleotide to form stable contacts through the mobilized polypeptide binding surface with GroES, creating a huge domed chamber that could house a folding protein (**Fig. 2b**). The hydrophobic binding surface was completely displaced from the cavity and replaced with a hydrophilic cavity lined with electrostatic residues. We inferred that, during this process, the polypeptide would be ejected from the hydrophobic surface into the newly formed GroES-encapsulated chamber. There, it could fold in solitary confinement, with no danger of aggregation. This was clearly the other major means by which the chaperonin provided kinetic assistance to protein folding. The GroEL-GroES chamber has been aptly referred to as an "Anfinsen cage"[27].

Implications

The discovery of chaperonins and their role in assisting protein folding in the cell has led to the recognition that folding is a closely monitored and regulated process that is central to cellular homeostasis. Folding has come to be seen as the necessary final step in the pathway from DNA to active, functional proteins. In addition to the chaperonins, multiple molecular machines have now been identified that participate in various aspects of folding. Some of them, such as the Hsp70 protein family, serve general functions, whereas others, such as Hsp90, have more specific roles. At the same time, protein unfolding has been shown to be an important process controlled by other chaperone proteins. And the concept of protein homeostasis—the balance between folding and unfolding, synthesis and degradation—has become a central theme in understanding how cells and organisms respond to changing environments and to stress and disease.

Even with this panoply of cellular-folding and -unfolding machines, it is clear that Anfinsen's 'thermodynamic hypothesis'—that the native structure of a protein is determined by its amino acid sequence—still holds. But as Anfinsen himself recognized, the kinetics of folding is crucial in the cellular milieu, where proteins are synthesized and folded on the time scale of seconds to minutes, in response to a similar time scale of physiologic and environmental changes. Just as Anfinsen, in his later studies, used protein disulfide isomerase to provide kinetic assistance to RNase refolding *in vitro*, changing recovery of its activity from an hours-long process to one requiring only a few minutes, so, too, do cells use chaperone proteins to kinetically control and direct the folding of proteins to their thermodynamically determined final state.

ACKNOWLEDGMENTS

Many of the major participants in the chaperonin work referred to above are pictured in the illustration on p. xiii. There have been many other collaborators, both in the early work and more recently, who also contributed substantially to the understanding of this system. I regret that space limitations prevented me from referring to them here, but I want to express how deeply grateful I am to everyone with whom I've interacted. Surely, the recognition of this work is shared by all of us. But, more selfishly, it has been a pure joy for me over these past 20 years to work in the lab, at the bench, day by day and side by side with my group members, sharing our ideas, dreams, reagents, frustrations and, of course, joys of discovery as a scientific family. No one could ask for a more enjoyable life. I wish to thank the US National Institutes of Health for supporting the early phase of our work and the Howard Hughes Medical Institute (HHMI) for supporting our subsequent work. I am particularly grateful to the HHMI for allowing me to 'follow my nose' through this work, no matter how risky the undertaking. I also thank HHMI for making our work environment a paradise in which to pursue ideas and do experiments. Finally, I thank W. Fenton for his critical comments during the preparation of this manuscript.

COMPETING FINANCIAL INTERESTS

The author declares no competing financial interests.

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