

Chaperone-assisted protein folding: the path to discovery from a personal perspective

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Protein folding is the process by which newly synthesized polypeptide chains acquire the three-dimensional structures necessary for biological function. For many years, protein folding was believed to occur spontaneously, on the basis of the pioneering experiments of Christian Anfinsen, who showed in the late 1950s that purified proteins can fold on their own after removal from denaturant¹. Anfinsen had discovered the fundamental principle that the linear amino acid sequence holds all the information necessary to specify a protein's three-dimensional structure. But it soon became apparent that test-tube folding experiments work mostly for small, single-domain proteins, often only in conditions far removed from those encountered in a cell. Large proteins frequently fail to reach native state under these experimental conditions, forming nonfunctional aggregates instead. Despite these problems, protein folding was of little interest to cell biologists until the mid- and late 1980s, when the chaperone story began to unfold. As a result, we now know that in cells, many (perhaps most) proteins require molecular chaperones and metabolic energy to fold efficiently and at a biologically relevant rate. Here I describe, from a personal perspective, the developments leading to this new view.

Setting the stage

After completing my doctoral thesis at Heidelberg University in 1985, I joined the laboratory of renowned biochemist Walter Neupert at the University of Munich. Walter's group studied how cell organelles such as mitochondria import newly synthesized proteins from the cytosol. The move to Munich turned out to be crucial for both my professional and personal future—the latter because

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Walter allowed me to attend a molecular biology summer school on a Greek island, where I met my future wife Manajit (facilitated by the chaperone-free, Mediterranean atmosphere, I might add).

Around the time of my arrival in Walter's department, it was becoming clear that proteins need to adopt an unfolded state to cross the mitochondrial double membrane², and definitive evidence for this came in 1986 from the group of Gottfried Schatz³. In 1988, two papers, one from Günter Blobel and the other from Randy Schekman and Elizabeth Craig, showed that the cytosolic precursors of mitochondrial and secretory proteins interact with so-called heat-shock proteins, such as Hsp70, before membrane translocation^{4,5}. These proteins had already been described in the 1970s, on the basis of their striking tendency to increase in abundance upon exposure of cells to heat stress⁶. Hugh Pelham had suggested in 1985 that these proteins are involved in dissociating protein aggregates that form under such stress conditions⁷. On the basis of the two 1988 papers, it was now plausible that Hsp70 also functioned in stabilizing precursor proteins in an unfolded state for translocation. How, then, would proteins fold after import into the mitochondria? The atmosphere was ripe for something new, and I found myself in the right place at the right time. I became completely fascinated by the possibility of using the mitochondrial system to study how proteins fold in a physiological environment.

The Hsp60 story

In pursuing the problem of protein folding, I was fortunate that Walter introduced me to Art Horwich from Yale, and this led to an exciting and very productive collaboration (Fig. 1). Art had conducted a genetic screen in yeast to identify cellular machinery involved in mitochondrial protein uptake. The first temperature-sensitive mutant we analyzed in



Figure 1 Art Horwich (right) and I (left) in March 1991 taking a walk near my parents' village in the northern part of the Black Forest. Photograph by Manajit Hayer-Hartl.

detail had a defect in the mitochondrial precursor protease⁸, but another mutant had a far more interesting and puzzling phenotype. The mitochondria of this mutant strain, called *mif4*, were still capable of importing and proteolytically processing proteins at the nonpermissive temperature, but the proteins failed to assemble into their respective oligomeric complexes⁹. This included the trimeric enzyme ornithine transcarbamylase and the β -subunit of the F1-ATPase. Other proteins that undergo complex intramitochondrial sorting, such as the Rieske Fe/S protein¹⁰, were incompletely processed. Interestingly, the *mif4* mutation mapped to the nuclear gene encoding the mitochondrial heat-shock protein Hsp60 (ref. 9), a protein that had just been identified as the

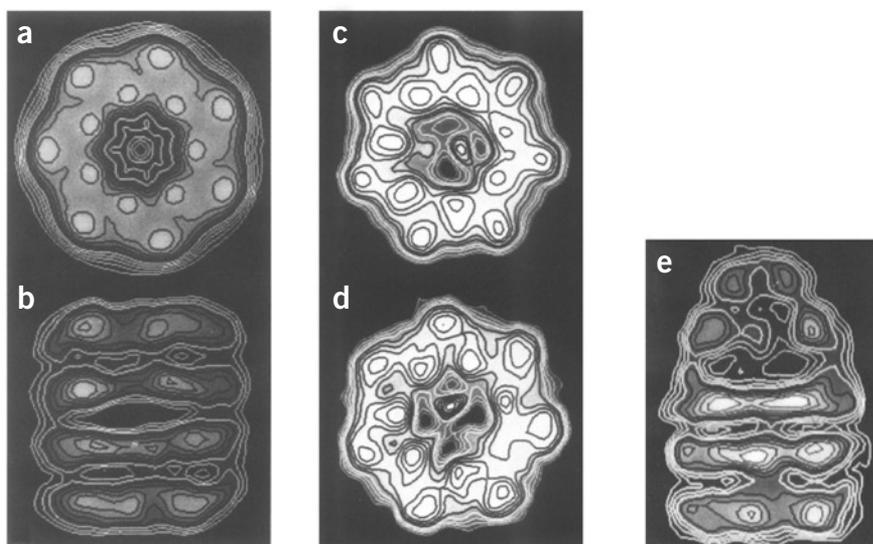


Figure 2 Averaged electron microscopic images. (a,b) GroEL alone. (c) GroEL-unfolded rhodanese. (d) GroEL-unfolded rhodanese-GroES. (e) GroEL-GroES complexes from the collaboration with Wolfgang Baumeister²². End-on views are shown in a, c and d and side views in b and e. In e, GroES sits like a lid on the GroEL cavity, causing a conformational change in the outer domains of the interacting GroEL subunits (also see ref. 31).

homolog of *Escherichia coli* GroEL and of the Rubisco large subunit-binding protein (RBP) of chloroplasts^{11,12}. These proteins were known to form large macromolecular complexes, for which John Ellis and Costa Georgopoulos had coined the name ‘chaperonin’¹² in 1988, to denote a special class of molecular chaperone¹³. *E. coli* GroEL had already been discovered in the 1970s to function with GroES as a host factor in phage assembly^{14–16}, and John Ellis had observed that large subunits of the enzyme Rubisco in chloroplasts interact with RBP before assembly¹⁷.

We interpreted our findings with *mif4* mitochondria in terms of a similar role for yeast Hsp60 in oligomeric assembly⁹. However, the discovery of the basic function of Hsp60 in polypeptide chain folding was just around the corner. To investigate its possible role in folding, we used the monomeric protein dihydrofolate reductase (DHFR) for import into mitochondria of the fungus *Neurospora crassa*¹⁸. Although denatured DHFR refolded spontaneously *in vitro*, this was not observed in mitochondria. Instead, we found that newly imported DHFR associated with Hsp60 in a highly protease-sensitive, unfolded state. Formation of folded, protease-resistant DHFR occurred concomitantly with release from Hsp60 in an ATP-dependent manner¹⁸. We thus concluded that the chaperonins mediated protein folding. Hence, the defects in oligomeric assembly observed in the *mif4* mitochondria resulted from the failure of protein subunits to fold. These findings in 1989 established the new paradigm of chaperone-assisted protein folding.

The quest for the chaperonin mechanism

The next obvious question was how, exactly, the chaperonins mediated folding. This question would keep several laboratories busy for years to come, and the path to discovery was fraught with ‘kinetic traps’. George Lorimer made the next advance by showing that bacterial Rubisco, a dimeric complex, can be reconstituted from a denatured state with the help of GroEL and GroES¹⁹. We set

up a similar system to analyze the assisted folding of two monomeric proteins²⁰, DHFR and rhodanese, the latter being highly aggregation prone under Anfinsen refolding conditions. Fluorescence spectroscopy revealed that GroEL binds its substrates in a loosely folded, ‘molten globule’-like conformation, exposing hydrophobic surfaces. As proteins in such states tend to aggregate, their binding by GroEL explained how aggregation is prevented. We also obtained evidence that at least partial folding occurred in association with GroEL and that this process was dependent on the presence of GroES, suggesting an encapsulation mechanism. Indeed, in his commentary accompanying our 1991 paper²⁰, Tom Creighton modeled the substrate protein into the GroEL cavity capped by GroES²¹.

In 1991, I took a faculty position at Memorial Sloan-Kettering Cancer Center in the newly established Department of Cellular Biochemistry and Biophysics led by Jim Rothman. (The year also marked the beginning of my long-term collaboration with Manajit Hayer-Hartl, an excellent biochemist.) Two key members of the Munich group, Jörg Martin and Thomas Langer, joined us in the New York adventure. We next investigated the GroEL system by electron microscopy in collaboration with Wolfgang Baumeister at the Max Planck Institute of Biochemistry in Munich²². It was already known that GroEL is an ~800-kDa complex consisting of two stacked, heptameric rings (Fig. 2a,b). The new images revealed that GroEL binds the

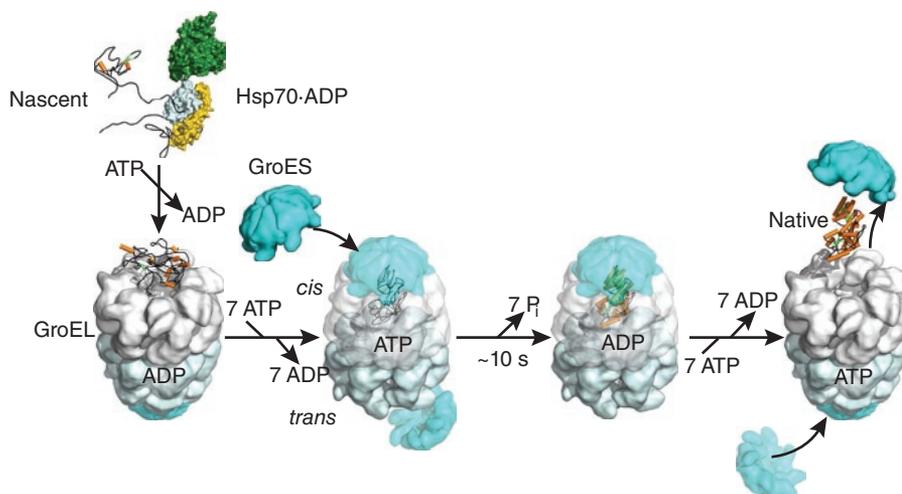


Figure 3 The current model for protein folding in the GroEL-GroES chaperonin cage. Substrate binding to GroEL (upon transfer from the upstream chaperone Hsp70; see Fig. 4) may result in local unfolding⁵⁷. ATP binding then triggers a conformational rearrangement of the GroEL apical domains. This is followed by the binding of GroES (forming the *cis* complex) and substrate encapsulation for folding. At the same time, ADP and GroES dissociate from the opposite (*trans*) GroEL ring, allowing the release of substrate that had been enclosed in the former *cis* complex (omitted for simplicity). The substrate remains encapsulated, free to fold, for the time needed to hydrolyze the seven ATP molecules in the newly formed *cis* complex (~10 s). Binding of ATP and GroES to the *trans* ring causes the opening of the *cis* complex. Diagram modified from ref. 58.

unfolded protein in the ring center (Fig. 2c,d), and Art Horwich obtained similar results independently²³. We also found that GroES, a heptameric ring of ~10 kDa subunits, binds like a lid over the central GroEL cavity, causing major conformational changes in the interacting GroEL subunits²² (Fig. 2e). The idea that the folding reaction might take place in the central cavity was soon reinforced. In 1993, we showed that the GroEL-GroES complex is asymmetrical and highly dynamic, with GroES binding and unbinding in a mechanism regulated by the GroEL ATPase²⁴. Importantly, Jörg Martin found that GroES can bind the same ring that holds the unfolded substrate protein. Suddenly, the pieces of the puzzle began to fall into place, and we became increasingly confident that GroEL and GroES essentially function as a folding cage^{24,25} (Figs. 3 and 4), later dubbed the “Anfinsen Cage” by John Ellis²⁶.

Nature’s solution to the problem of protein folding in the crowded cellular environment seemed extremely impressive in its simplicity and elegance: a single protein molecule folding in a macromolecular cage would be unable to aggregate. However, our evidence to support this model was still indirect²⁴, and many researchers had difficulty accepting the idea of a proteinaceous folding cage. Hence, an alternative model, in which folding occurred in bulk solution during jumps of the protein between GroEL complexes^{27,28}, was more appealing. GroEL was thought to unfold kinetically trapped folding intermediates, affording them another chance at spontaneous folding in solution. Unfortunately for us, the crystal structure of GroEL, solved in 1994 by the late Paul Sigler in collaboration with Art Horwich²⁹, seemed to support the folding-in-solution model³⁰: the central cavity of GroEL was simply not wide enough for even a relatively small protein such as rhodanese to fit. However, this interpretation of the structure failed to take into account that GroEL cooperates with GroES. Interestingly, a month before the crystal structure was published, Helen Saibil and her colleagues had shown by advanced electron cryomicroscopy that GroES binding causes a dramatic conformational change in GroEL, resulting in the formation of a large space capped by GroES³¹. We interpreted this finding to be in support of the folding-cage model.

Definitive evidence for folding inside the GroEL-GroES cage was published by Mark Mayhew³² in my group and by Jonathan Weissman³³ in Art Horwich’s group in 1996. Different approaches were used, but the conclusion was clear: ATP-dependent GroES binding results in protein displacement and encapsulation in the central cavity (the so-

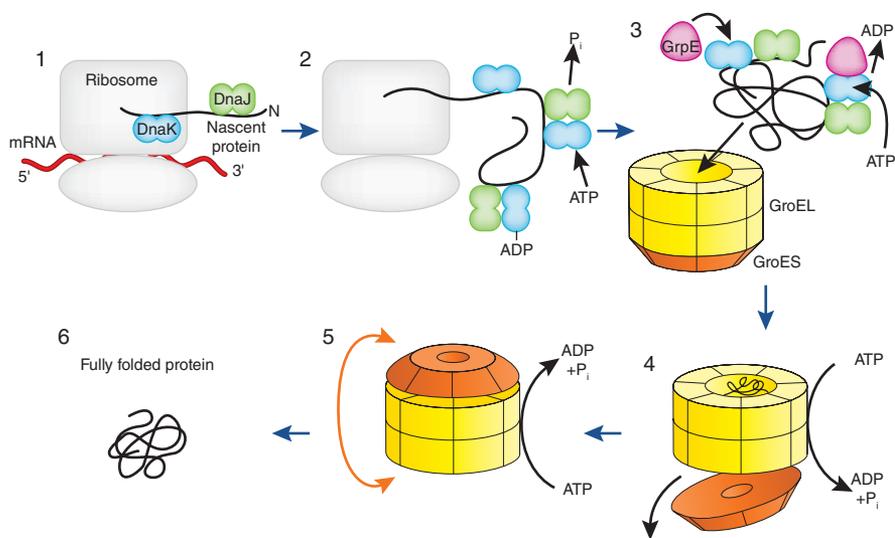


Figure 4 Model from 1993 for the pathway of chaperone-assisted protein folding in the *E. coli* cytosol, shown for a GroEL-dependent protein (reproduced from ref. 25). The nascent chain is stabilized in a folding-competent state during translation by the Hsp70 chaperone system (DnaK, DnaJ) (1 and 2). These chaperones bind hydrophobic segments exposed by the extended chain that will later be buried within the folded structure. Upon completion of translation, the protein is unable to fold using the Hsp70 chaperone system and must be transferred into the central cavity of GroEL. This step requires GrpE, the nucleotide exchange factor of DnaK (3). After binding of the protein in a molten globule-like conformation into the open ring of GroEL (4), the protein is encapsulated by GroES in the folding cage (5). Folded protein emerges from the cage as GroES unbinds (6). The model was later extended to include the cooperation of DnaK with the ribosome-bound chaperone trigger factor and the finding that the Hsp70 system mediates the folding of proteins that do not require the physical environment of the chaperonin cage^{47,55}.

called *cis* complex). The cage opens again after ~10 s in a reaction timed by the allosterically regulated GroEL ATPase³⁴: When the seven ATP molecules in the GroES-bound GroEL ring have been hydrolyzed, ATP binds to the *trans* ring³⁵, triggering the signal that causes GroES to unbind (Fig. 3). The crystal structure of the GroEL-GroES complex in 1997 provided a detailed view of the folding cage³⁶. It confirmed the previous suggestion³⁷ that GroES binding must cause the burial of the hydrophobic residues on GroEL used for substrate binding, rendering the cage hydrophilic and thus permissive for folding.

The function of GroEL and GroES is essential³⁸, and it therefore was of major interest to identify the *E. coli* proteins that require the chaperonin for folding^{39,40}. Using quantitative proteomics in collaboration with Matthias Mann, we showed in 2005 that at least 250 different proteins interact with GroEL upon synthesis (~10% of cytosolic proteins)⁴¹. Of these, 60–80 proteins are absolutely GroEL-GroES-dependent, including a number of essential proteins^{41,42}. They are generally below ~60 kDa in size and can be accommodated by the chaperonin cage. Interestingly, many GroEL-dependent proteins have complex fold topologies comprising a mixture of α -helices and β -sheets, such as the TIM barrel,

and are known for their tendency to populate kinetically trapped states during folding. We suggested that the confining environment of the chaperonin cage not only prevents aggregation but also can smooth rugged folding-energy landscapes, allowing folding to occur within a biologically relevant time frame⁴³.

A chaperone relay for protein folding

As the GroEL story developed, we were also busy trying to resolve another puzzle. In the late 1980s, it became clear that cells contain at least one other type of ATP-dependent chaperone, Hsp70, which also interacts with newly synthesized proteins. But what was the relationship between the Hsp70 and chaperonin systems, and did they cooperate in protein folding? There was evidence that Hsp70 binds hydrophobic peptides⁴⁴ and can associate with nascent polypeptide chains emerging from ribosomes⁴⁵, that is, at a stage when the polypeptide is structurally incomplete and not yet capable of folding. Taking this into consideration, we envisioned a coherent pathway in which Hsp70 would interact with the (growing) polypeptide chain, preventing premature misfolding and aggregation (the negative principle), and then GroEL-GroES would mediate folding of the completed protein to the native state (the positive principle)^{46,47}.

To test this hypothesis, we attempted to reconstitute the reaction pathway with pure components. Costa Georgopoulos had just shown in 1991 that *E. coli* Hsp70, called DnaK, cooperates with two additional proteins, DnaJ and GrpE⁴⁸, and we were generously provided with the plasmids and purification protocols for the three proteins by the late Hatch Echols in Berkeley. I still remember vividly our excitement when Thomas Langer in my lab had purified all the proteins and was ready to begin with these important experiments. Upon dilution of denatured rhodanese into buffer containing DnaK, DnaJ and ATP, we observed efficient prevention of aggregation, but the protein did not fold, even when GroEL and GroES were added to the reaction. Strikingly, addition of GrpE, the nucleotide exchange factor of DnaK, catalyzed the transfer of the unfolded protein from DnaK-DnaJ to GroEL-GroES for folding⁴⁶ (Fig. 4). This pathway was later confirmed for other GroEL-dependent proteins both *in vivo* and *in vitro*^{41,49}.

The reconstitution experiments provided several interesting insights: the Hsp70 system is as efficient as GroEL in preventing protein aggregation, efficient binding of unfolded protein by DnaK (Hsp70) requires ATP and regulation by DnaJ (Hsp40), DnaJ also functions as a chaperone on its own and GrpE is necessary for ATP-dependent cycles of protein binding and release from Hsp70. Experiments with increasingly unfolded versions of the model protein α -lactalbumin showed further that Hsp70 binds preferentially to the extended polypeptide chain, whereas GroEL prefers the collapsed molten globule state⁴⁶, thus ordering the two chaperone systems along the cellular folding pathway. In collaboration with Bernd Bukau, we found soon after that the DnaK system also assists folding through cycles of protein binding and release^{50,51}. This mechanism is used by 20% or more of cytosolic proteins⁴⁹ but does not work for the set of proteins that populate kinetically trapped intermediates and that depend on transfer into the protected environment of the chaperonin cage for folding⁴¹.

In 1994, Judith Frydman in my lab extended the principle of a sequential chaperone pathway to the eukaryotic cytosol⁵², where we found that Hsp70 cooperates with the previously characterized chaperonin TRiC⁵³. We also found that, in this system, the chaperones allow larger multidomain proteins to begin to fold during translation in a domain-wise fashion⁵². This mechanism serves to prevent nonproductive interactions between folding domains, providing the biological solution to the protein-size problem encountered in the Anfinsen folding experiment^{52,54}. The basic organization of the cytosolic chaperone pathway

has been highly conserved in evolution, with very similar implementations found in bacteria, archaea and eukarya⁵⁵.

Outlook

Over the past two decades, the chaperone field has developed into a highly active and rapidly expanding branch of molecular life sciences. Although the basic functions of chaperones in protein folding are now firmly established, we are only beginning to appreciate the vital role of cooperative chaperone networks in maintaining cellular protein homeostasis and proteome integrity. Understanding these processes at the systems level will be of far-reaching medical relevance, as the number of diseases linked to protein misfolding and aggregation is growing steadily and already includes type II diabetes, Alzheimer's disease, Parkinson's disease and many others. According to recent studies, each of these diseases manifests with an age-dependent decline in cellular chaperone capacity⁵⁶. The field is entering a new phase of excitement fueled by the hope that drugs can be developed to reset the chaperones in our cells to a more youthful state, postponing the onset of disease.

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COMPETING FINANCIAL INTERESTS

The author declares no competing financial interests.

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