Unfolding the chaperone story

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ABSTRACT **Protein folding in the cell was originally assumed to be a spontaneous process, based on Anfinsen's discovery that purified proteins can fold on their own after removal from denaturant. Consequently cell biologists showed little interest in the protein folding process. This changed only in the mid and late 1980s, when the chaperone story began to unfold. As a result, we now know that in vivo, protein folding requires assistance by a complex machinery of molecular chaperones. To ensure efficient folding, members of different chaperone classes receive the nascent protein chain emerging from the ribosome and guide it along an ordered pathway toward the native state. I was fortunate to contribute to these developments early on. In this short essay, I will describe some of the critical steps leading to the current concept of protein folding as a highly organized cellular process.**

It is an honor to share the E. B. Wilson Medal with Art Horwich. I have fond memories of our close collaboration and of the excitement we felt when our experiments provided first evidence of protein folding as a chaperone-assisted process. These early findings would have a lasting impact on our scientific careers.

I was introduced to research as a young medical student at Heidelberg University. My doctoral thesis in the biochemistry department under the guidance of Wilhelm Just focused on the functions of peroxisomes in the rat liver. One of our findings was that the peroxisomal membrane system could be induced by thyroid hormones. I was lucky that Walter Neupert at Munich University was invited as an external reviewer of my thesis, and this led to my joining his group in 1985. Walter was famous for his studies on how mitochondria import newly synthesized proteins from the cytosol. His mentorship turned out to be critical, not only professionally but also personally, as he allowed me to attend a molecular biology summer school where I met my future wife, Manajit. Incidentally, I am writing these lines on our 30th wedding anniversary.

Around the time of my arrival in the Munich lab it became clear that proteins had to be unfolded in order to translocate from the cytosol across the mitochondrial membranes. How then would these proteins fold and assemble inside the organelle? I found myself at the right place at the right time to address this fascinating problem.

PROTEIN FOLDING IN MITOCHONDRIA

I was fortunate that Walter put me in touch with Art Horwich (Figure 1), who had conducted a genetic screen in yeast to identify cellular machinery involved in mitochondrial protein import. One of his temperature-sensitive mutants, called *mif4* (for mitochondrial import function 4), was particularly puzzling. We embarked on an exciting collaboration and found that *mif4* mitochondria remained import-competent, but the imported proteins failed to assemble into their respective oligomeric complexes (Cheng *et al.*, 1989). Intriguingly, the *mif4* mutation mapped to the nuclear gene encoding mitochondrial Hsp60, the homologue of *Escherichia coli* GroEL and the Rubisco subunit-binding protein (RBP) of chloroplasts—large complexes called "chaperonins" (Hemmingsen *et al.*, 1988). GroEL was known as a genetic host factor in phage propagation, and RBP had been observed to bind unassembled subunits of the enzyme Rubisco (Barraclough and Elis, 1980), suggesting a role in mediating protein assembly.

While our initial findings on Hsp60 were consistent with such a role, a second generation of experiments soon revealed the basic function of the chaperonin in polypeptide chain folding. In these experiments, carried out with my student Joachim Ostermann, we targeted the monomeric protein dihydrofolate reductase (DHFR) to mitochondria (Ostermann *et al.*, 1989). Denatured DHFR will refold spontaneously in vitro, but strikingly, this was not what we observed in mitochondria. Instead, the newly imported protein associated with Hsp60 in an unfolded state that was stabilized under ATP limiting conditions. Formation of folded DHFR occurred upon readdition of ATP concomitantly with release from Hsp60. We concluded that Hsp60—and by analogy the other chaperonins—mediated protein folding. Hence the defects in oligomeric assembly observed in the

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Abbreviations used: DHFR, dihydrofolate reductase; Hsp, heat-shock protein.

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

mif4 mitochondria resulted from the failure of protein subunits to fold. These findings in 1989 established the new paradigm of chaperone-assisted protein folding (reviewed in Hartl, 1996).

THE CHAPERONIN FOLDING CAGE

But how did the chaperonins work? George Lorimer made the next advance by reconstituting bacterial Rubisco, a dimeric enzyme, from the denatured state with the help of GroEL and its cofactor GroES (Goloubinoff *et al.*, 1989). To be sure that we measured folding, not assembly, Jörg Martin in my lab chose monomeric proteins (DHFR and rhodanese) as substrates for reconstitution experiments (Martin *et al.*, 1991). Fluorescence spectroscopy revealed that GroEL binds nonnative proteins in a loosely folded, "molten globule"–like conformation, preventing their aggregation. In the presence of GroES, GroEL released the substrate protein in a more folded, less aggregation-prone state.

In 1991 I took on a faculty position at Sloan-Kettering Cancer Center in Manhattan in the new department of Jim Rothman. We next investigated the GroEL system by electron microscopy in collaboration with Wolfgang Baumeister (Langer *et al.*, 1992a). As shown earlier, the ~800 kDa GroEL complex consists of two stacked, heptameric rings. The new images revealed that unfolded substrate protein binds in the ring center. We also observed that GroES, a heptameric ring of ~10 kDa subunits, bound like a lid over one end of the GroEL cylinder, concomitant with major conformational changes in the interacting GroEL ring. The GroEL–GroES complex turned out to be highly dynamic, with GroES undergoing cycles of binding and release in a manner regulated by the GroEL ATPase (Martin *et al.*, 1993). Jörg Martin also found that during this reaction, GroES transiently bound to the GroEL ring holding the protein substrate—indirect evidence that GroES encapsulated the protein in the GroEL cavity. Definitive evidence that folding occurs inside the GroEL–GroES cage was obtained 3 years later by Mark Mayhew in my group and Jonathan Weissman in the Horwich lab (Mayhew *et al.*, 1996; Weissman *et al.*, 1996). Both studies concluded that encapsulation in the central GroEL cavity allows a single protein molecule to fold unimpaired by aggregation (Figure 2). Art Horwich's and Paul Sigler's crystal structure of the GroEL–GroES complex provided a detailed view of the folding cage (Xu *et al.*, 1997).

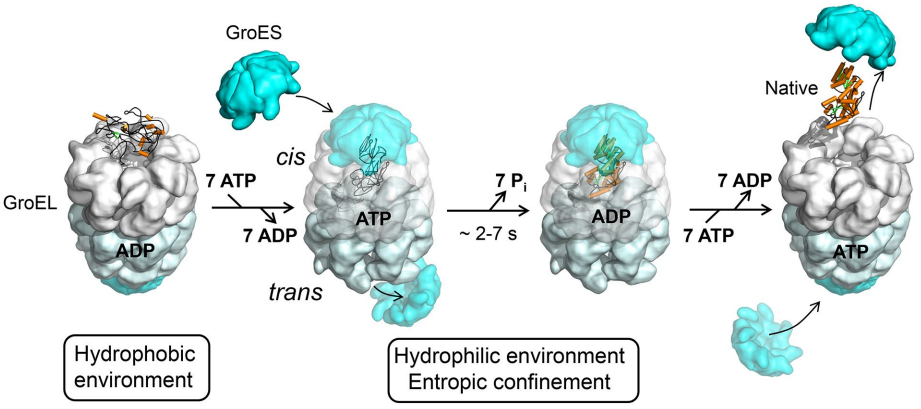

FIGURE 2: The GroEL/GroES reaction cycle. The current model for protein folding in the chaperonin cage is shown. Substrate binding to GroEL may result in local unfolding (Sharma *et al.*, 2008). ATP binding then triggers a conformational rearrangement of the GroEL apical domains. This is followed by 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). Substrate remains encapsulated, free to fold, for the time needed to hydrolyze the seven ATP molecules in the newly formed *cis* complex (~2–7 s, dependent on temperature). Folding inside the cage may be accelerated due to entropic confinement of dynamic folding intermediates. Binding of ATP and GroES to the *trans* ring causes the opening of the *cis* complex. Diagram reproduced from Hartl *et al.* (2011).

The function of GroEL and GroES is essential, but the natural substrates of the chaperonin system remained unknown. We analyzed the flux of proteins through GroEL (Ewalt *et al.*, 1997) and identified ~250 GroEL substrates (~10% of cytosolic proteins) by proteomics (Houry *et al.*, 1999; Kerner *et al.*, 2005). A subset of these proteins proved to be absolutely dependent on GroEL/GroES for folding. These proteins are generally below ~60 kDa in size and fit into the chaperonin cage. They have complex fold topologies, such as the TIM barrel, and tend to populate aggregation-prone folding intermediates.

More recently, in collaboration with Manajit Hayer-Hartl, we discovered that substrate encapsulation in the chaperonin cage not only serves to prevent aggregation but also markedly (up to 100-fold) accelerates the folding of some proteins over their spontaneous folding rate (measured in the

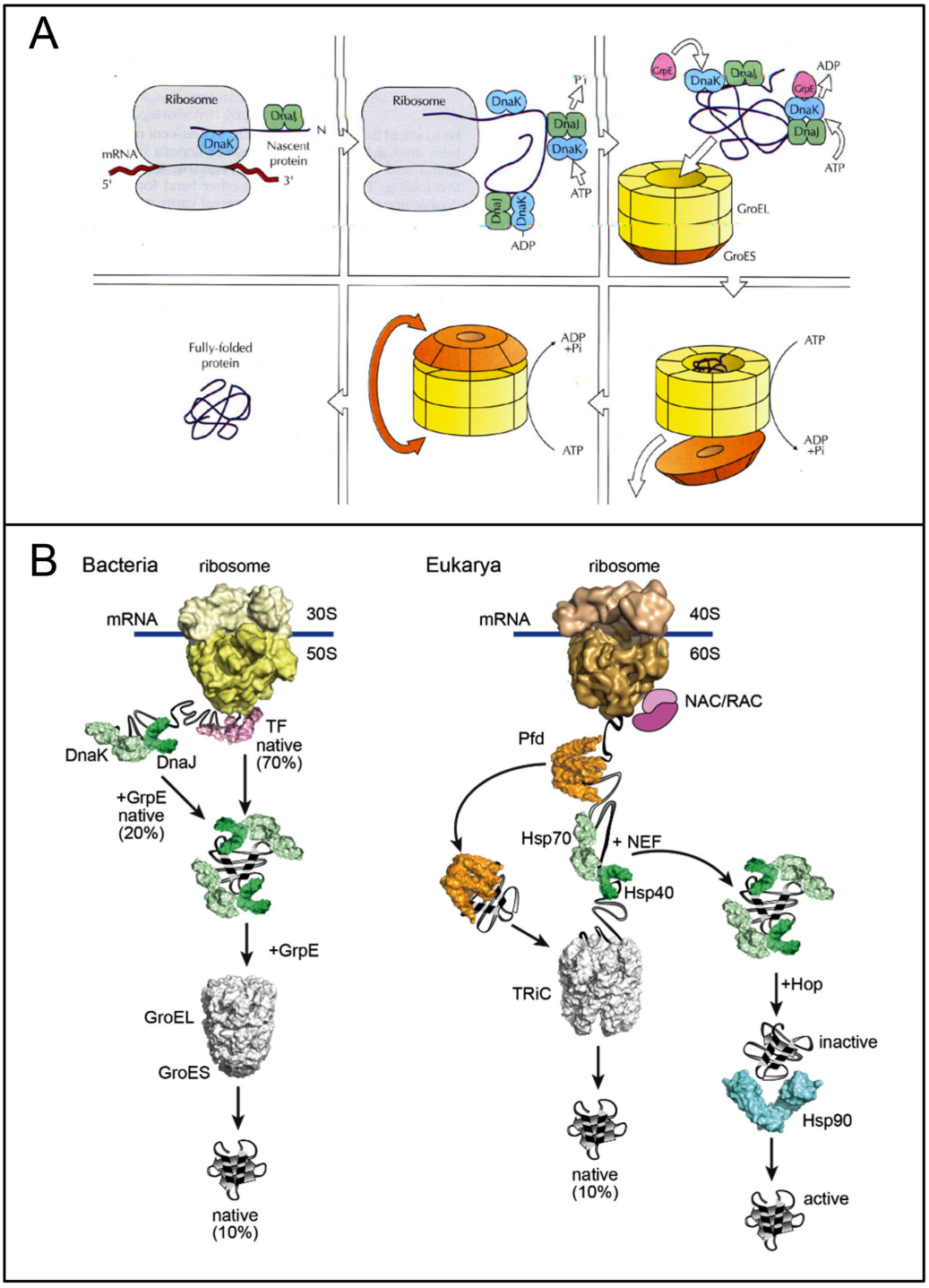

FIGURE 3: Chaperone pathways in the bacterial and eulkaryotic cytosol. (A) Early model for the *E. coli* cytosol, shown for a GroEL-dependent protein (reproduced from Martin and Hartl, 1993). In clockwise direction: The nascent chain is stabilized in a folding-competent state during translation by the Hsp70 chaperone system (DnaK/DnaJ). 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 with DnaK and DnaJ and must be transferred into the central cavity of GroEL. This step requires GrpE, the nucleotide exchange factor of DnaK. Following binding of the protein in a "molten globule"–like conformation into the open ring of GroEL, the protein is encapsulated by GroES in the folding cage. The model was later extended to include the cooperation of DnaK with the ribosome-bound chaperone Trigger factor (Deuerling *et al.*, 1999; Teter *et al.*, 1999) and the finding that the Hsp70 system mediates the folding of proteins that do not require the physical enmvironment of the chaperonin cage (Szabo *et al.*, 1994). (B) Current models for the folding pathways in the bacterial and eukaryotic cytosol reproduced from Balchin *et al.* (2016). NAC/RAC are eukaryotic ribosome-binding chaperones with a function similar to that of bacterial Trigger factor (TF). Prefoldin (Pfd) recruits the chaperonin TRiC to certain nascent chains. Like TRiC, the Hsp90 chaperone system functions downstream of Hsp70. Hop mediates contacts between Hsp70 and Hsp90.

absence of aggregation; Brinker *et al.*, 2001; Georgescauld *et al.*, 2014). We attribute this to an effect of confinement that facilitates the conversion of dynamic folding intermediates to the native state by lowering the entropic component of the folding energy barrier (entropic confinement; Tang *et al.*, 2006; Chakraborty *et al.*, 2010). This function of the cage is important in allowing folding to occur on a biologically relevant time scale.

CHAPERONE PATHWAYS

While we studied the chaperonins, significant progress was made by Jim Rothman and others in understanding another chaperone class, the Hsp70s. Evidence emerged that Hsp70s bind hydrophobic peptides (Flynn *et al.*, 1989) and can associate with nascent polypeptide chains during translation (Beckmann *et al.*, 1990), that is, at a stage when the polypeptide is structurally incomplete and not yet able to fold. Taking this into consideration, we envisioned the existence of a coherent pathway in which Hsp70 would interact first with the (growing) polypeptide chain, maintaining it in a nonaggregated, folding competent state, followed by GroEL/GroES-assisted folding of the completed protein. To test this hypothesis, we attempted to reconstitute the proposed chaperone pathway with pure components. The Georgopoulos group had just shown that the ATPase of the *E. coli* Hsp70, DnaK, was regulated by two additional proteins, DnaJ and GrpE (Liberek *et al.*, 1991). Key to our success in demonstrating the chaperone function of Hsp70 was to include these factors in the reconstitution experiments. Thomas Langer in the lab made several seminal observations (Langer *et al.*, 1992b): Upon dilution of denatured rhodanese into buffer containing DnaK/DnaJ and ATP, rhodanese aggregation was efficiently prevented, but the protein did not fold, even when GroEL and GroES were added. Strikingly, addition of GrpE, the nucleotide exchange factor of DnaK, catalyzed the transfer of the unfolded protein from DnaK/DnaJ to GroEL/GroES for folding. We later confirmed this pathway for other GroEL-dependent proteins, both in vivo and in vitro (Teter *et al.*, 1999; Kerner *et al.*, 2005; Calloni *et al.*, 2012).

Thomas Langer's reconstitution experiments showed that DnaK (Hsp70) binds unfolded proteins efficiently, but only when combined with DnaJ (Hsp40) in the presence of ATP. We also observed that DnaJ functioned as a chaperone on its own and GrpE was necessary for ATP-dependent cycles of protein binding and release from Hsp70. Experiments with model proteins showed further that Hsp70 binds extended polypeptides, whereas GroEL prefers collapsed molten globule-like states, thus ordering the two chaperone systems along the folding pathway. Collaborative studies with Bernd Bukau revealed that the DnaK system also assists folding through cycles of protein binding and release (Schröder *et al.*, 1993; Szabo *et al.*, 1994). This mechanism is utilized by 20% or more of cytosolic proteins, but fails for the set of proteins that rely on the protected environment of the chaperonin cage for folding.

In 1994, Judith Frydman in the lab extended the principle of a sequential chaperone pathway from bacteria to the eukaryotic cytosol (Frydman *et al.*, 1994; Frydman and Hartl, 1996). Here Hsp70 cooperates with the chaperonin TRiC, which Judith had previously characterized (Frydman *et al.*, 1992). She also discovered that larger multidomain proteins begin to fold during translation in a domain-wise manner, in close association with chaperones. This mechanism serves to avoid nonproductive interactions between folding domains, as shown by Bill Netzer, and thereby solves the problem of folding large proteins (Netzer and Hartl, 1997). The basic organization of the cytosolic chaperone pathway has been highly conserved in evolution, with very similar implementations found in bacteria, archaea, and eukarya (Figure 3).

OUTLOOK

Over the past two decades the chaperone field has developed into a highly active and rapidly expanding branch of molecular life sciences. We are beginning to appreciate the critical 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 numerous diseases are linked to protein misfolding and aggregation, including type II diabetes, Alzheimer's, Parkinson's, and many others. There is a clear vision that molecular chaperone research will soon enter into the exciting phase of clinical applications.

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