Protein Folding and Chaperones

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Advanced article

Article Contents

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Proteins fold via specific pathways to achieve their native structure. Protein structures are, however, inherently unstable, hence folding and unfolding are in equilibrium. Protein instability is a major concern inside the cell. Specialised proteins called molecular chaperones are, therefore, required to assist proteins in folding and to prevent aggregation of folding intermediates. Many different classes of chaperones are conserved throughout all kingdoms of life, many of which are known as heat shock proteins. Chaperones typically recognise hydrophobic patches, but the exact functions and mechanisms of action of the various chaperone classes are very different. The main chaperone classes Hsp70, Hsp90, Hsp100 and chaperonins all depend on ATPase cycles, whose activities are fine-tuned by co-chaperones. The molecular understanding of the mechanism of both chaperones and protein folding are key problems in present-day life sciences and molecular medicine.

Introduction

Nascent polypeptide chains fold into three-dimensional protein structures determined by their amino acid sequence (Anfinsen, 1973). Potentially, a polypeptide chain could adopt an enormously large number of different conformations because most bonds can rotate freely. It would take a time longer than the age of the universe to sample all of these, searching for the native fold (Levinthal, 1969). Some small proteins, however, fold on a sub-millisecond timescale (Daggett and Fersht, 2003). The folding process is thus not a random search for the native structure. Instead, folding follows pathways, which sample only a limited number of possible conformations (Dill and Chan, 1997).

Two main pathways that are not mutually exclusive have been discovered: the nucleation-condensation mechanism and the framework mechanism (Daggett and Fersht, 2003). Nucleation-condensation refers to the initiation of folding at a nucleus, the secondary structure of which is stabilised by long-range tertiary interactions arising when the rest of the polypeptide chain folds around it. Secondary and tertiary structures thus form simultaneously in this most universal folding mechanism. Some proteins consisting of only α-helices, however, fold via the framework mechanism (Mayor *et al*., 2003): folding starts with formation of the secondary structural elements, after which the pathway can proceed efficiently towards the native three-dimensional structure.

From a thermodynamic point of view, the folding process along those pathways can be visualised by an energy funnel (**Figure 1**) (Dill and Chan, 1997). The pathway progresses to conformations with a lower level of free energy until the stable native state has been reached. In this process, the number of possible conformations and thus the entropy decreases. Notably, the native structure is not necessarily the conformation with the lowest free energy level. For some proteins oligomers or aggregates might be more stable, hence lower in free energy (Clark, 2004; Englander *et al*., 2007). The state the polypeptide reaches is probably not solely determined by the free energy level but rather a consequence of the pathway it follows. Both thermodynamic and kinetic factors define the pathway, avoiding local energy minima or aggregated states from which the polypeptide cannot proceed to the native state. **See also**: **Molten Globule**; **Protein Folding: Overview of Pathways**

Protein Structures Are Labile

The three-dimensional protein structure is stabilised by non-covalent interactions: hydrogen bonds, electrostatic interactions

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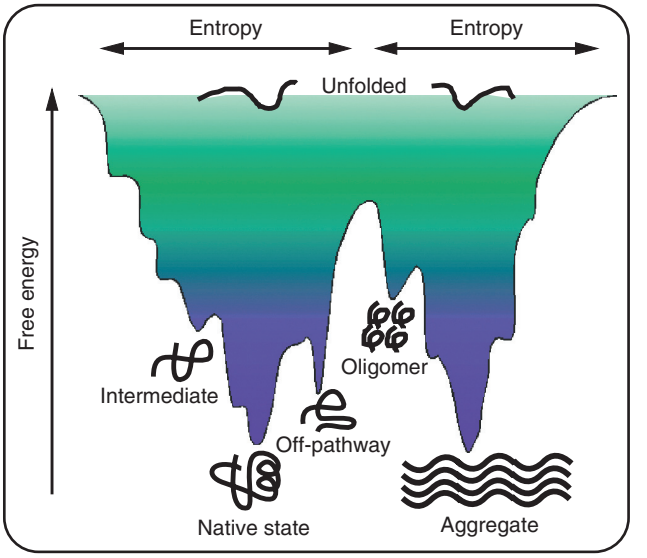

Figure 1 **Energy landscape describing protein folding and aggregation**. The unfolded polypeptide chain moves towards conformations with lower free energy. In this process, the number of available conformations, hence entropy, decreases. The landscape consists of two funnels: one leading to the native state of the protein, the other to an aggregate. Alternatively, the polypeptide may become trapped in a local energy minimum, such as a partially folded state or an oligomer.

and hydrophobic interactions. Together these interactions weigh up against a loss of entropy that is caused by folding of the polypeptide chain. However, the free energy difference between the folded and unfolded state of a typical protein is minimal, only 20–60 kJ mol^{-1}, corresponding to the energy of just one to three hydrogen bonds (Fersht and Daggett, 2002). This implies that a folded protein can readily unfold again, and minor changes to the protein or its environment are sufficient to initiate that.

Protein folding is thus not a one-way process: folding and unfolding are in equilibrium. In a series of landmark experiments, Anfinsen and coworkers found that chemically denatured proteins could refold *in vitro*, without requiring any additional factors (Anfinsen, 1973). This proved that folding and unfolding are reversible processes.

However, not all proteins fold. Many proteins contain domains that are intrinsically disordered and function without having a defined three-dimensional structure–6% of the *Escherichia coli* and 31% of the mouse proteome is unstructured (Oldfield and Dunker, 2014). Still, interaction with a binding partner induces formation of structure in some cases, as happens to some eukaryotic transcription factors when binding to DNA. Also post-translational modifications can lead to formation of defined structural elements, as exemplified by the kinase activation loop, which becomes ordered and competent to bind substrates only after activation by phosphorylation. These phenomena illustrate that protein folding is a dynamic equilibrium that can be affected by many factors. **See also**: **Intrinsically Disordered Proteins**

Protein Folding Inside the Cell

The situation inside the cell differs significantly from the conditions in *in vitro* folding studies. Anfinsen did his classical experiments with small single-domain proteins in diluted solutions. Contrastingly, the cell is densely packed with macromolecules, the total protein concentration typically exceeding 300 g L^{-1}. This creates excluded volume effects, which dramatically restrict diffusion inside the cell (Ellis, 2007). This has significant consequences for protein folding, unfolding and aggregation. Folding is supported because unfolded, extended states require more space and are, therefore, less favourable. On the other hand, the reduction of available volume increases a protein's effective concentration, which may promote aggregation. But the presence of other proteins also provides charged and polar surfaces, which stabilise a folded protein, while damaged proteins, in contrast, may offer unwanted hydrophobic surfaces that promote protein aggregation. **See also**: **Protein Folding *In Vivo***

Despite fundamental differences, knowledge obtained by *in vitro* experiments is highly relevant for understanding protein folding inside the cell. Several studies comparing both situations showed that many aspects of folding are similar. For example, the energy difference between folded and unfolded protein *in vivo* was found to be very close to that *in vitro* (Ignatova and Gierasch, 2004). Structural studies have found that the three-dimensional structure is virtually the same whether in a test tube or inside a cell (Sakakibara *et al*., 2009). Strikingly, the flexibility of proteins inside the cell is higher than in diluted solutions *in vitro*. This at first glance surprising result might be explained by conformational changes induced by binding of partner proteins or

ligands, but maybe even by polar interactions with the surfaces of surrounding proteins.

Why Is Protein Folding in the Cell Chaperoned?

Several fundamental processes in the cell require that proteins fold or unfold (Becker *et al*., 2012; Rapoport, 2007). The life of proteins *in vivo* is, therefore, rather more dynamic than *in vitro*. Maintenance of protein homeostasis is essential for life, a process known as proteostasis (Hipp *et al*., 2014; Morimoto and Cuervo, 2014). Proteins have to fold initially when being synthesised at the ribosome. Folding has to be prevented or a folded protein unfolded for transfer through narrow pores into different compartments. Proteins have to form complexes with sometimes varying partners, while complexes with unwanted partners or uncontrolled aggregation have to be prevented. At the end of its life, every protein has to be unfolded for degradation. This picture is significantly more complex than folding and unfolding experiments in the test tube using denaturants. Each cell owns a proteostasis network to control the fate of its proteins (Hipp *et al*., 2014; Morimoto and Cuervo, 2014). The major players in this network are a specific set of proteins, the molecular chaperones. **See also**: **Quality Control of Protein Folding in the Cytosol**

Anfinsen demonstrated that the protein structure is determined by the primary sequence. Therefore, chaperones are not expected to influence the structure of the folded protein. However, it might not be impossible that chaperones may channel the folding pathway of some (multi-domain) proteins by lowering a specific energy barrier to a local minimum, leaving the energy barrier to the global minimum unchanged.

Chaperones can refold proteins both *in vivo* and *in vitro*. For the first time this was shown for luciferase from firefly, which was refolded by *E. coli* chaperones, the DnaK Hsp70 system (Schröder *et al*., 1993). Remarkably, it is a bacterial chaperone system that refolds a peroxisomal protein from a eukaryotic organism. Protein folding is a fundamental process, and chaperone activity does not necessarily stop at the species barrier. In many cases, however, recombinantly produced proteins fail to fold, indicating that proteome and chaperonome in each cellular compartment are adapted to each other.

Why Are There so many Different Chaperones?

Historically, chaperone activity was seen as ability to prevent aggregation. Aggregation is particularly dramatic upon large-scale unfolding of proteins following a sudden increase in temperature. Almost all organisms react to this by expressing heat shock proteins, many of which are molecular chaperones. Later on it was found that these proteins are also expressed under normal conditions, albeit at lower levels, but still many molecular chaperones are named 'heat shock proteins', Hsp's. **See also**: **Heat Shock Response**

Chaperones appear in different classes. The main classes are conserved from bacteria to humans and are named according to their apparent molecular weight: Hsp100, Hsp90, Hsp70 and Hsp60; they are also known as 'chaperonin' (Saibil, 2013). These different chaperone families do not share sequence similarities and differ dramatically in shape and structure (**Figure 2**). They have in common that they are all triggered by an ATPase cycle. The members of these main chaperone classes are often accompanied by co-chaperones that regulate activity, control the ATPase cycle or target protein substrates. **See also**: **Heat Shock Proteins (HSPs): Structure, Function and Genetics**

Many of the conserved families are represented in several compartments of the cell. The most widespread chaperone families are the Hsp70s and Hsp90s. They occur in all main folding compartments of the higher eukaryotic cell, the cytosol, mitochondria and the endoplasmic reticulum, but also in the cytosol of most bacteria.

In addition to those main classes, several other specialist chaperones that are ATP-independent are found in some organisms or compartments, such as small heat shock proteins, trigger factor, Skp or calnexin.

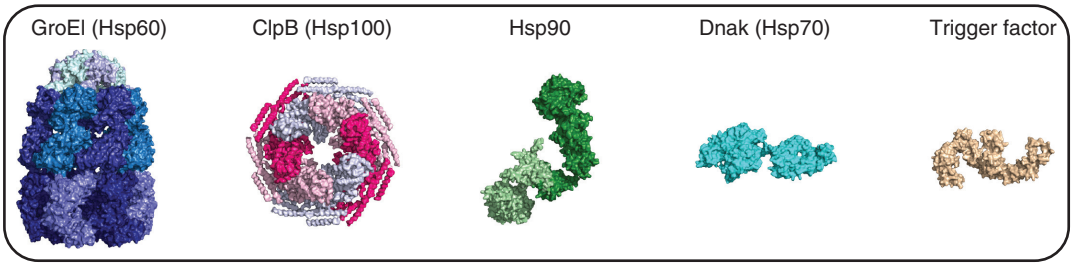

Figure 2 **Chaperones in the bacterial and eukaryotic cytosol**. The ATP-dependent chaperones of the *E. coli* and eukaryotic cytosol and the nascent chain binding trigger factor are shown in surface representation. The molecules are shown to scale. GroEL (in complex with GroES 7 mer), Hsp60 family, oligomeric state 14mer, pdb file 1aon; ClpB, Hsp100, hexamer, 1qvr; Hsp90, dimer (Karagöz *et al*., 2014); DnaK, Hsp70, monomer, 2kho; trigger factor, no eukaryotic homologues, monomer, 1w26. Nota bene: all chaperones undergo conformational changes upon binding nucleotide, co-factors and/or substrate. Pictures were made using Pymol.

Chaperone Families Differ in Structure and Function

The substrate specificity of the molecular chaperones defines 'unfolded protein' from the cell's point of view. The main chaperone classes all share a preference for hydrophobic amino acids, which are typically inside a folded protein (Winkler *et al*., 2012a). Both the binding modes of the different chaperone families and their mechanisms of action, however, differ fundamentally.

The first encounter of the newly synthesised protein with the chaperonome of the cell is just outside the exit channel of the ribosome. A problem for newly synthesised polypeptide chains is that the speed of protein folding exceeds that of translation. Therefore, the synthesised part of the polypeptide chain starts folding already before translation is completed. Because the C-terminal part of the chain is not yet available, the emerging domain cannot reach its native conformation, constituting an aggregation-prone intermediate. The cell must overcome this problem to obtain natively folded proteins and inhibit misfolding and aggregation (**Figure 3**). In *E. coli*, one of the ATP-independent chaperones, the ribosome-bound chaperone trigger factor, welcomes the nascent chain with a hydrophobic dome over the exit site, which is thought to shield the hydrophobic surface of the emerging protein until synthesis is finished and the domain may fold into a native structure (Merz *et al*., 2008). In the eukaryotic cell, the functional

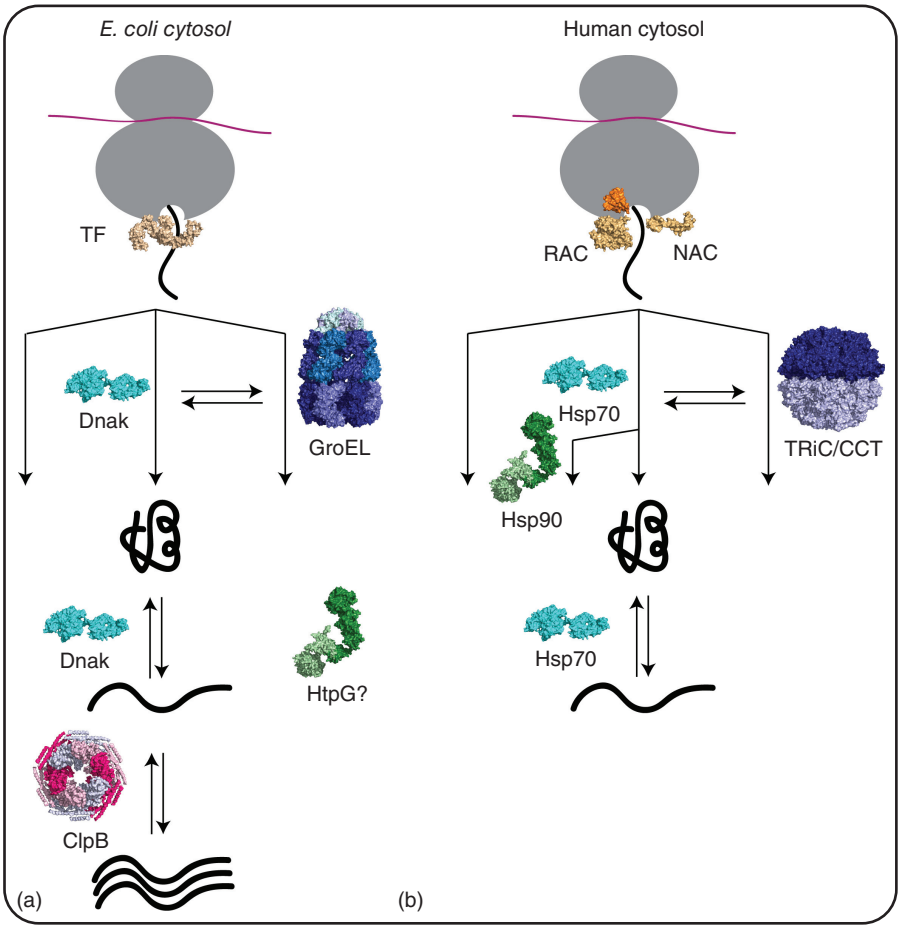

Figure 3 **Protein folding in the cytosol, assisted by ATP-driven machines**. (a) *Protein folding in the cytosol of E. coli.* The nascent chain emerges from the ribosome, where it meets its first chaperone, trigger factor (TF). TF is ATP-independent but profits from the ATP-driven growth of the polypeptide by the ribosome. Proteins may then either fold on their own, or assisted by the Hsp70 system DnaK and its co-factors (not shown), or assisted by the chaperonin, GroEL (shown in complex with its co-factor GroES). Nascent polypeptides may travel between chaperonin and DnaK in case they first bind to a chaperone system that fails in folding them. Proteins may unfold again, in particular under stress conditions, which may lead to aggregation. Even large aggregates can be reversed by concerted action of the DnaK system and the Hsp100 chaperone ClpB. The general action of the bacterial Hsp90 homologue HtpG remaines largely elusive. (b) *Protein folding in the human cytosol.* The processes are similar to those in *E. coli*, with the following exceptions: (1) The eukaryotic ribosome does not have a trigger factor homologue but functional analogues nascent chain associated complex (NAC; pdb 1tr8) and RAC (pdb 4gni for the Ssz1 ATPase domain in orange and 4gmq for Zuo1 in red). (2) Hsp90 is essential for folding of a subset of substrate, often following Hsp70 action. (3) There is no Hsp100 chaperoning activity. Chaperone pictures are based on the pictures in **Figure 2**. Human Hsp70 is depicted as its *E. coli* counterpart and NAC is represented by the archaeal homologue. TRiC/CCT (pdb 4v94) is the eukaryotic Hsp60 family member. Co-chaperones are omitted, except for the GroEL-GroES complex.

analogues of trigger factor, nascent-chain-associated complex (NAC) and ribosome-associated complex (RAC), shield the ribosomal exit site, but other folding factors, including Hsp70 family members, are also found there (Preissler and Deuerling, 2012).

Hsp70 chaperones are monomers that provide only a short substrate binding cleft (Mayer, 2013). They associate with short hydrophobic stretches of up to five amino acids long that are particularly enriched in leucine (Rüdiger *et al*., 1997). Leucine is the most abundant of the large hydrophobic residues, which is why Hsp70 chaperones will find binding sites in almost every protein that is unfolded. The versatility of Hsp70 is emphasised by their sheer number – there are several homologues in the human cytosol. Strikingly, most Hsp70s are not involved in folding of nascent proteins; in other words, most chaperones are not involved in initial folding (Preissler and Deuerling, 2012). Hsp70s also have special functions such as controlling the activity of heat shock transcription factors, and one homologue in *E. coli*, HscB, has the sole function to support the incorporation of an Fe-S cluster into one specific protein, IscU (Vickery and Cupp-Vickery, 2007).

In contrast to the small Hsp70s, the chaperonin is a large double-doughnut-shaped complex that encloses its substrate (Saibil *et al*., 2013; Xu *et al*., 1997). Representatives of this family are the 14meric GroEL in *E. coli*, which acts with the 7meric co-factor GroES, and the 16meric CCT in the eukaryotic cytosol (Saibil *et al*., 2013). They associate with folding intermediates by exposing a hydrophobic surface. After binding, substrates are buried from the solvent within a hydrophobic dome, allowing protein folding secluded from other proteins (Chen *et al*., 2013). It is remarkable, however, that only a minority of proteins, fewer than 1 in 10, use the sophisticated chaperonins to fold (Kim *et al*., 2013). The chaperonin mechanism is described in more detail elsewhere. **See also**: **Chaperonins**

From all chaperones, only one family is able to dissolve larger aggregates, the Hsp100s (Glover and Lindquist, 1998). They entirely unfold their substrates with the help of Hsp70s and pull them through the narrow central hole of their hexameric ring (Winkler *et al*., 2012a). It is interesting to note that there is no Hsp100 activity in either the cytosol or the endoplasmic reticulum of higher organisms. It is, however, present in the cytosol of yeast and *E. coli*, and in mitochondria. Environmental changes are more drastic for unicellular organisms, so they have to cope with a larger risk for protein aggregation than the cells of higher eukaryotes.

For many chaperones the folding mechanism is unknown, one of them being the dimeric Hsp90 machine. In the eukaryotic cytosol, Hsp90 is thought to be involved in later stages of protein folding and activation, but the function of the Hsp90 homologues in the endoplasmic reticulum, mitochondria and the bacterial cytosol is unclear (Li *et al*., 2012). Despite their high concentration, which suggests an important role, deletions do not have any phenotype, probably because other chaperones can take over. To give another example, the *E. coli* Hsp70 homologue DnaK can cover up for a deletion of trigger factor (Preissler and Deuerling, 2012). This highlights that the redundancy of the chaperonome makes it difficult to pinpoint the specific role of the individual chaperone.

A Chaperoning Paradigm: The Hsp70 Machine

A key paradigm to explain chaperone activity is the Hsp70 system. Hsp70 chaperones occur in two states (**Figure 4**). The ATP-bound state binds unfolded proteins at very fast on- and off-rates; the ADP state binds the substrate at very slow on- and off-rates, locking in the substrate (Mayer, 2013). The activity of the chaperone requires fast binding, to be able to compete with fast aggregation of unfolded proteins, but also tight binding. The Hsp70 system overcomes this problem by associating with the substrate with the fast on-rate of the ATP state, and then hydrolysing ATP to keep the substrate bound with the slow off-rate of the ADP state. Release of the ADP allows rebinding of ATP, moving Hsp70 to the fast off-rate state, stimulating release of the bound substrate. How these steps promote protein folding is not fully understood. One model is that the Hsp70s act in cycles of binding and release: after leaving the Hsp70 the substrate may fold and Hsp70 will not re-bind. If it does not succeed in folding, it will enter the Hsp70 binding cleft for another cycle.

The Hsp70 cycle allows for two tuning possibilities. One is stimulation of ATP hydrolysis, the other is nucleotide exchange (Mayer, 2013). Hsp70 systems use these tuning possibilities for being recruited to specific subsets of substrates but also for linking the chaperone machine to specific functions. For example, one of the nucleotide exchange factors, Bag1, binds to the proteasome and links Hsp70 to the degradation machine. ATP hydrolysis is stimulated by J proteins, which allows Hsp70s to bind to substrates with high affinity. Even more J co-chaperones than Hsp70 homologues exist in the folding compartments, for example, 13 in the yeast cytosol, constituting a subtly regulated network (Kampinga and Craig, 2010).

The Hsp70 system cooperates with other chaperones, which provide complementary binding modes. In the bacterial cytosol, folding intermediates travel between the Hsp70 chaperone DnaK and the chaperonin GroEL, if the first choice chaperone does not succeed. The eukaryotic cytosol organises for some Hsp70 substrates, for example, steroid receptors, transfer to the Hsp90 system via the adaptor protein Hop (Li *et al*., 2012). In contrast to the narrow Hsp70 binding site, Hsp90 offers an elongated binding site that extends over a length of up to 100 Å (Karagöz *et al*., 2014). Hydrophobic contacts are scattered over this side, so that only large proteins with a mild exposure of hydrophobic residues can effectively make use of this (**Figure 5**). This property is typical for late folding intermediates, but it is also shared by some intrinsically disordered proteins. One example is the protein Tau, the aggregation of which causes Alzheimer's disease (Karagöz *et al*., 2014).

Protein Folding, Chaperones and Disease

Many diseases are caused by perturbations in the balance between protein folding and unfolding. For example, cancer patients often

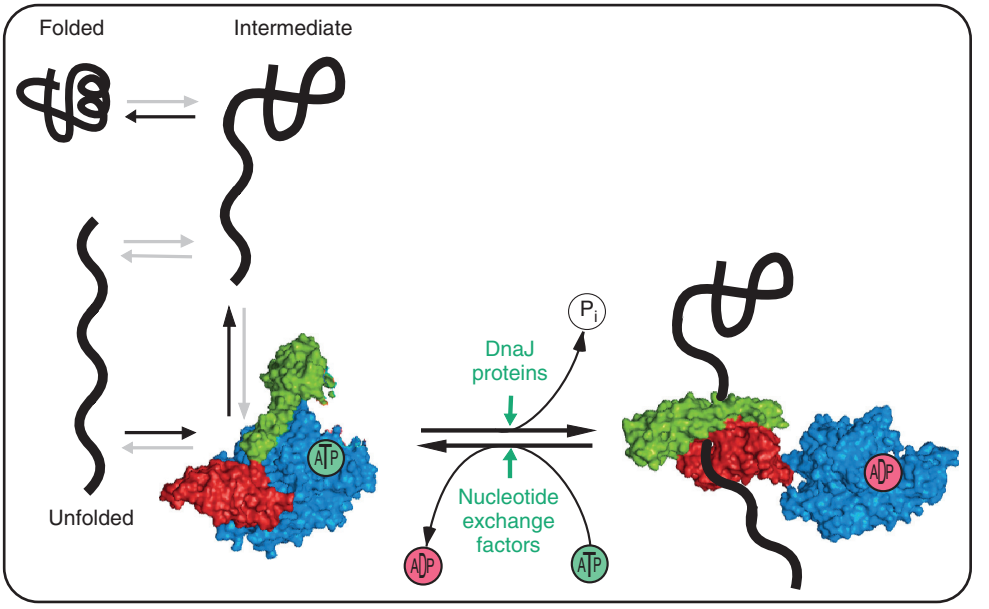

Figure 4 **The Hsp70 ATPase cycle, as the substrate sees it**. Hsp70 consist of an ATPase domain (blue) and a substrate binding domain that has a substrate holding segment (red) and a lid segment (green). ATP binding triggers opening of the substrate binding domain, ATP hydrolysis encloses the substrate. Release of ADP and rebinding of ATP opens Hsp70, leading to release of the substrate that subsequently may fold into the native state. It is not known whether the substrate has a different structure after Hsp70 release than before Hsp70 interaction. Hsp70 chaperone activity is tuned by stimulating ATP hydrolysis by J proteins and by triggering nucleotide exchange. Protein folding/unfolding processes that are not assisted by Hsp70 are indicated by grey arrows, all other processes are indicated by black arrows. The pictures are based on pbd file 2kho of DnaK for the closed ADP conformation and on pdb file 2qxl of yeast Hsp70 sse1 for the open ATP conformation.

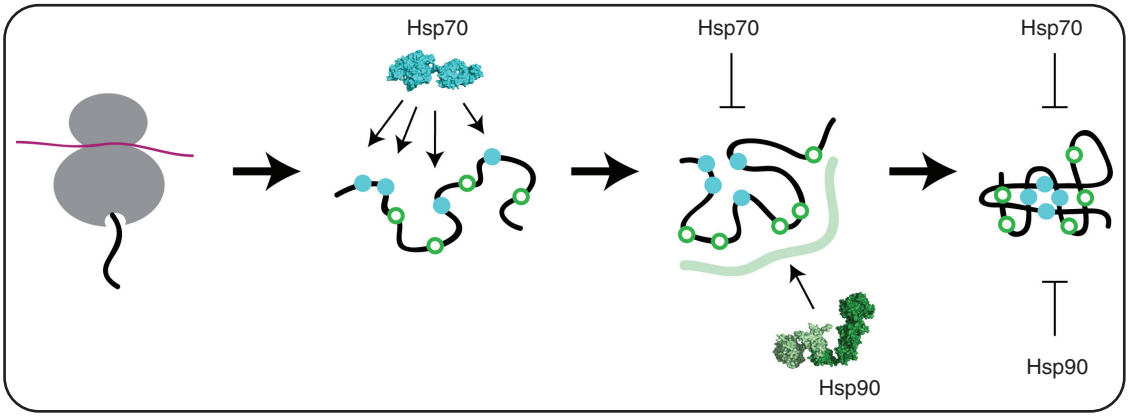

Figure 5 **Recognition of hydrophobic residues by Hsp70 and Hsp90 during the folding pathway**. Directly after translation, the unfolded polypeptide exposes hydrophobic residues that are recognised by Hsp70. In later stages of folding, these form the hydrophobic core of the protein, leaving Hsp70 unable to bind, but exposing scattered hydrophobic residues that allow binding of Hsp90. Finally, the protein adopts its correct fold and does not present surfaces for chaperone binding anymore.

harbour mutations in the genes encoding tumour suppressor proteins such as p53 (Joerger and Fersht, 2007). Mutations destabilise the protein structure and shift the equilibrium between native and nonnative state towards the unfolded, inactive form. In prion diseases, such as Creutzfeldt-Jakob, unfolded proteins misfold to adopt an infectious conformation that leads to aggregation and cell death (Knowles *et al*., 2014). Protein aggregation is also associated with neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Knowles *et al*., 2014). It is one of the major challenges in the field to understand the role of the chaperonome in these processes. Chaperones protect against protein misfolding and aggregation, but they are also captured by protein aggregates, which may disturb chaperone function in the cell (Kim *et al*., 2013).

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