

Chaperone machines for protein folding, unfolding and disaggregation

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Abstract | Molecular chaperones are diverse families of multidomain proteins that have evolved to assist nascent proteins to reach their native fold, protect subunits from heat shock during the assembly of complexes, prevent protein aggregation or mediate targeted unfolding and disassembly. Their increased expression in response to stress is a key factor in the health of the cell and longevity of an organism. Unlike enzymes with their precise and finely tuned active sites, chaperones are heavy-duty molecular machines that operate on a wide range of substrates. The structural basis of their mechanism of action is being unravelled (in particular for the heat shock proteins HSP60, HSP70, HSP90 and HSP100) and typically involves massive displacements of 20–30 kDa domains over distances of 20–50 Å and rotations of up to 100°.

Autophagy

A process in which intracellular material is enclosed in a membrane compartment and delivered to the lysosome (vacuole in yeast) for degradation and recycling of the macromolecular constituents.

Unfolded protein response

(UPR). A signalling system that regulates the balance between folding capacity of the endoplasmic reticulum (ER) and protein synthesis. If misfolded proteins accumulate, this pathway triggers apoptosis.

Heat shock proteins

(HSPs). The expression of these proteins is greatly enhanced by increased temperature or other stress conditions. Most chaperones are HSPs.

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Protein quality control, also known as proteostasis, constitutes the regulation of protein synthesis, folding, unfolding and turnover. It is mediated by chaperone and protease systems, together with cellular clearance mechanisms such as autophagy and lysosomal degradation. These quality control systems have an essential role in the life of cells, ensuring that proteins are correctly folded and functional at the right place and time^{1,2}. They are crucial for mitigating the deleterious effects of protein misfolding and aggregation, which, by unclear mechanisms, can cause cell death in neurodegeneration and other incurable protein misfolding diseases (BOX 1). A set of protein families termed molecular chaperones assists various processes involving folding, unfolding and homeostasis of cellular proteins. After protein denaturation caused by stress (for example, due to heat or toxin exposure) or disease conditions, proteins can be unfolded, disaggregated and then refolded, or they can be targeted for disposal by proteolytic systems. Found in all cellular compartments, chaperones act on a broad range of non-native substrates. The endoplasmic reticulum (ER), in particular, is a major site for protein production and quality control in membrane and secretory systems. If it is overburdened by misfolded proteins, the unfolded protein response (UPR) triggers cell death by apoptosis³.

Chaperones are not typical macromolecular machines with a well-defined substrate. The major molecular chaperones (TABLE 1) have little specificity but provide essential assistance to a complex and highly specific process, protein folding^{4,5}. How do they assist folding or unfolding of

diverse proteins? Most of the main chaperones use cycles of ATP binding and hydrolysis to act on non-native polypeptides, facilitating their folding or unfolding⁶. Others simply have a 'handover' role, protecting nascent subunits during the assembly process. Some ATP-dependent chaperones, also known as protein remodelling factors, mediate targeted disassembly, unfolding or even reversal of aggregation. Because of the disordered nature of unfolded, partially folded or aggregated proteins, structural details are lacking for the interactions between chaperones and their protein substrates.

An emerging functional feature of chaperones is their highly dynamic behaviour. Despite the great importance and utility of X-ray crystal structures, the resulting atomic structures can give a misleading impression of static, fixed conformations. It seems that the conformations of these ATPases are only weakly coupled to their nucleotide states (that is, whether they are bound to ATP, ADP or in the unbound state) and that they are in a continual state of rapid fluctuation.

This Review focusses on the roles and mechanisms of representatives of the major families of general, ATP-dependent chaperones, namely the heat shock proteins (HSPs; also known as stress proteins) HSP60, HSP70, HSP90 and HSP100. We summarize our current understanding of these allosteric machines and address the ways in which the energy of ATP binding and hydrolysis are used to unfold misfolded polypeptides for either refolding or disaggregation. These considerations underlie a key unanswered question: which protein conformations have chaperones evolved to prevent (under conditions

Box 1 | Protein misfolding diseases

Mutations that destabilize a protein can cause the loss of protein function. If the protein is degraded and aggregation is prevented, serious pathological consequences may be avoided. However, the aggregation of misfolded proteins creates toxicity (toxic gain of function). Simple loss-of-function mutations in CFTR (cystic fibrosis transmembrane conductance regulator) destabilize the protein, leading to its misfolding in the endoplasmic reticulum (ER) and subsequent degradation, but they do not cause cell death. Conversely, retinitis pigmentosa mutations in the highly abundant photoreceptor protein rhodopsin affects its folding and transport and eventually result in photoreceptor cell death and blindness^{111,112}.

Serious neurodegenerative conditions, including Alzheimer's disease, Parkinson's disease, Huntington's disease and prion disease, result from the aggregation of a diverse set of peptides and proteins associated with the conversion to amyloid-like fibrillar assemblies. Although neurodegenerative diseases present an obvious burden in ageing societies, systemic conditions involving amyloids such as type II diabetes are equally serious. The common structural feature of amyloid is its cross β -fold in which the protein, whatever its native structure, is converted into a largely or wholly β -strand form. Short strands stack into ribbons that wind into fibrils with the strands running perpendicular to the fibril axis^{113–115}.

Although the structural and mechanistic basis of cytotoxicity remain obscure, there is evidence for membrane damage by oligomeric intermediates in amyloidogenesis, in addition to overload of protein quality control systems. In healthy individuals, chaperones prevent or rescue cells from pathological consequences by promoting refolding, degradation or sequestration into non-toxic aggregates^{116–119}.

The insulin-like signalling pathways that regulate lifespan provide a link between ageing and loss of proteostasis capacity^{1,2}. The role of chaperones in these processes has prompted efforts to chemically modulate these systems, with the goal of providing global protection against protein misfolding^{120,121}.

of stress or in misfolding diseases), that is, what is the nature of the cytotoxic species that result when protein homeostasis fails?

Chaperone families

Members of the HSP60 (known as GroEL in *Escherichia coli*), HSP70 (known as DnaK in *E. coli*), HSP90 (known as HptG in *E. coli*) and HSP100 (known as ClpA and ClpB in *E. coli*) families (the number indicates the molecular mass of each HSP subunit) interact either with aggregation-prone, non-native polypeptides or with proteins tagged for degradation.

HSP70 coordinates cellular functions by directing substrates for unfolding, disaggregation, refolding or degradation. HSP90 integrates signalling functions, acting at a late stage of folding of substrates that are important in cellular signalling and development and targeting substrates for proteolysis. By contrast, HSP60 acts at early stages of folding and provides an outstanding example of a highly coordinated and symmetric allosteric machine for protein folding. HSP100 is a sequential 'threading' machine for unfolding that cooperates with either a protease ring for degradation or HSP70 for disaggregation, thus avoiding the toxic effects of aggregation.

The mechanisms of action and allostery of the HSP60 and HSP70 families are understood in some detail. HSP60 forms symmetrical, self-contained complexes in which the substrate- and nucleotide-binding sites are located inside cavities, and they act in a concerted and global way on the substrate. By contrast, HSP70 exposes regulatory surfaces and cooperates with various binding proteins that can redirect its activity. It acts locally on unfolded regions of the substrate polypeptide.

HSP70 and HSP90 are highly interactive, functioning with many partners and cofactors. Conversely, HSP60 and HSP100 are 'loners'. They have few interacting partners and their active sites are not exposed on the outer surface of the protein complex. Despite their very different modes of action, these general chaperones share the common property of binding various non-native proteins to prevent their aggregation.

HSP70 — a tuneable chaperone system

HSP70 is the most abundant chaperone and exists as many orthologues in different cellular compartments. In association with various cofactors it carries out diverse functions, including protein folding, translocation across organelle membranes and disaggregation of aggregates. HSP70 has two domains: an ATPase domain and a substrate-binding domain. Its activity depends on dynamic interactions between these two domains and also on interactions between these domains and co-chaperones such as the HSP40 proteins (also known as J proteins, named after *E. coli* DnaJ) and nucleotide exchange factors (NEFs, which stimulate ADP release and nucleotide exchange after ATP hydrolysis)^{6–8}.

Cellular functions of HSP70. Even transient binding of an extended segment of a polypeptide chain to HSP70 could prevent misfolding and aggregation and maintain the substrate in an unfolded state for translocation to another cellular compartment. Indeed, the HSP70 system is an important component of the organelle translocation system on both sides of the membrane. The conformational cycle of HSP70 is used both for delivery of the substrate protein to the translocase that transports it across the organelle membrane and to capture or pull on the translocated polypeptide (reviewed in REF. 9). Regarding folding from the unfolded state, it seems likely that polypeptides can collapse into their native fold in free solution upon release from HSP70. Failure to reach the correctly folded state would lead to re-binding. Thus, the role of HSP70 in folding seems to be stabilizing the unfolded state or unfolding proteins until they can spontaneously fold upon reaching their correct cellular destination¹⁰.

In addition to its role in folding, HSP70 has other specific cellular functions. For example, together with auxilin (which is also a J protein co-chaperone), it disassembles the clathrin coat on membrane vesicles after completion of clathrin-mediated endocytosis¹¹. It also cooperates with HSP100 ATPases in disaggregating large aggregates (see below). The corresponding partner of HSP70 for disaggregation and/or detoxification of aggregates in the cytosol of higher eukaryotes has recently been identified as the NEF HSP110, which also has chaperone activity^{12–15}.

Structural basis of HSP70 function. The atomic structures of the ATPase domain and the substrate-binding domain of HSP70 were determined separately in the 1990s. Unexpectedly, the ATPase domain was found to have the same fold as actin and hexokinase, with two flexible domains surrounding a deep, nucleotide-binding

Allosteric machines

Macromolecular complexes in which the activity is indirectly modulated by binding of an effector at a site remote from the active site. This induces shifts in the domain or subunit structure that influence the conformation of the active site.

Amyloid

Protein species that form deposits consisting of fibrillar protein aggregates rich in β -sheet structure. They assemble from proteins that have unfolded or misfolded. About 20 distinct protein species are associated with particular amyloid diseases.

Table 1 | **ATP-dependent chaperones, examples of their cofactors and functions**

Chaperones*	Cofactors	Functions
Chaperonins		
HSP60 (also known as CPN60), GroEL (<i>Escherichia coli</i>), CCT (mammals), thermosome (archaea)	HSP10 (also known as CPN10), GroES, prefoldin	Protein folding, prevention of aggregation
HSP70 system		
DnaK (<i>E. coli</i>), Ssa, Ssb (<i>Saccharomyces cerevisiae</i>), BiP (also known as GRP78) (mammals; ER)	HSP40, DnaJ, Sis1, Hdj1, NEFs, GrpE, HSP110	Unfolding, disaggregation, stabilization of extended chains, translocation across organelle membranes, folding, regulation of the heat-shock response, targeting substrates for degradation
HSP90 system		
HptG (<i>E. coli</i>), GRP94 (ER)	HOP, p50, AHA1, p23, FKBP52, UNC45	Binding, stabilization and maturation of steroid receptors and protein kinases, delivery to proteases, buffer for genetic variation, regulation of substrate selection and fate, myosin assembly
HSP100		
ClpA, ClpB, ClpX, HslU (bacteria; mitochondria and chloroplasts), p97, RPT1–RPT6 (eukaryotic)	HSP70 system, ClpP, ClpS	Unfolding, proteolysis, thermotolerance, resolubilization of aggregates, remodelling

AHA1, activator of HSP90 ATPase 1; BiP, binding immunoglobulin protein; CCT, chaperonin-containing TCP1 complex; ER, endoplasmic reticulum; FKBP52, 52 kDa FK506-binding protein; GRP78, 78 kDa glucose-regulated protein; HSP, heat shock protein; HOP, HSC70–HSP90-organizing protein; NEFs, nucleotide exchange factors.*The species and/or localization is specified in brackets.

cleft that closes around ATP^{16,17} (FIG. 1a). The substrate-binding domain is thin and brick-shaped with a cleft capped by a mobile α -helical lid. Both the lid and the cleft open to allow substrate binding, which can then be trapped by the closing lid¹⁸ (FIG. 1a). The nucleotide state of the ATPase domain affects the opening (stimulated by ATP binding) and shutting (after ATP hydrolysis) of the substrate-binding site. However, the two domains, which are connected by a flexible linker, are not seen together in most crystal structures. The flexible linker, located at the base of the two domains remote from the cleft opening, is a key site in allosteric regulation.

A first view of the domain interaction came from the structure of yeast Sse1 (a homologue of mammalian HSP110)¹⁹. Although Sse1 and HSP110 are structural homologues of HSP70, they act as HSP70 NEFs. More recently, the crystal structure of a disulphide-trapped form of ATP-bound DnaK (which is the *E. coli* homologue of mammalian HSP70), together with methyl transverse relaxation optimized spectroscopy (methyl TROSY) nuclear magnetic resonance (NMR) and mutational probing of the domain association in a mutant deficient in ATP hydrolysis revealed how the HSP70 domains interact^{20–22}. Unlike Sse1 or HSP110, DnaK is highly dynamic, with rapid fluctuations between the docked and free conformations in all nucleotide-bound states.

A remarkable feature of the domain-docked complex is the intimate association of the substrate-binding domain with the ATPase domain. The substrate-binding domain is almost turned inside-out to wrap around the ATPase domain, with an extremely open orientation of its helical lid and a scissor-like motion of its β -subdomain that opens up the peptide-binding cleft (FIG. 1b). It has been proposed that the HSP70 mechanism of action involves several key steps. First, allosteric signalling from ATP binding and closure of

the nucleotide-binding cleft creates a binding site on the ATPase domain for the interdomain linker, which then recruits the substrate-binding domain. This domain docking distorts the substrate-binding cleft and opens the lid, which then binds to a different part of the ATPase domain^{20–22}.

Regulation by co-chaperones. HSP70 acts together with two co-chaperones in protein folding, namely an HSP40 and a NEF. The HSP40 family is very diverse, with many specialized members targeting HSP70 to specific sites or functions⁷. HSP40 is thought to act as the primary substrate recruiter for HSP70 and stimulates the HSP70 ATPase. For pathways involving nonspecific protein folding and refolding, the general HSP40 is an elongated, V-shaped dimer containing the characteristic, helical J domain that activates the HSP70 ATPase by binding at or near the interdomain linker^{23,24}. The J domain is followed by a disordered, Gly-Phe-rich region, two tandem β -subdomains and a dimerization domain²⁵. One of the β -subdomains contains a surface-exposed substrate-binding site. It seems likely that hydrophobic segments of a substrate polypeptide, following their initial recruitment to the shallow, accessible binding sites of HSP40, are delivered to the deeper channel of HSP70 for binding via the polypeptide backbone, with the J domain stimulating the ATPase^{26,27,8}. Thus, J proteins interact with both the nucleotide- and substrate-binding domains of HSP70, with flexibly linked sites stimulating the ATPase and delivering the bound polypeptide. NEFs such as *E. coli* GrpE or eukaryotic HSP110 interact near the entrance to the nucleotide cleft, moving the HSP70 subdomain IIB (FIG. 1a) and opening the cleft for nucleotide exchange^{28–30}. Although these interactions have been observed separately, how the dynamic complex functions as a whole has not yet been shown.

Methyl transverse relaxation optimized spectroscopy (methyl TROSY). A method that uses selective isotope labelling of methyl groups on protein side chains with a transverse relaxation scheme optimized for methyl groups to obtain well-resolved nuclear magnetic resonance (NMR) spectra from large protein structures far beyond the normal range obtained in NMR structure determination.

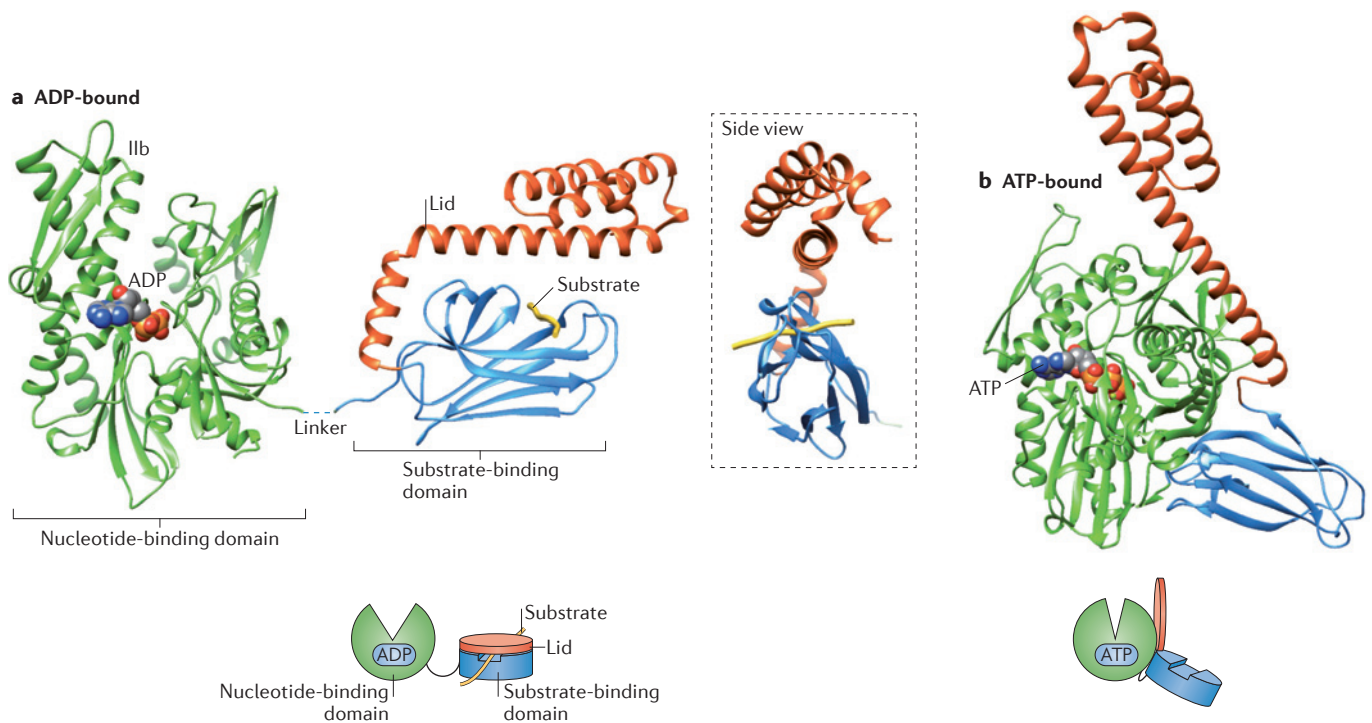


Figure 1 | HSP70 assemblies. **a** | In the ADP-bound or nucleotide-free state, the nucleotide-binding domain (green; Protein Data Bank (PDB) code: 3HSC)¹⁶ of heat shock protein 70 (HSP70) is connected by a flexible linker to the substrate-binding domain (blue; PDB code: 1DKZ), with the lid domain (red) locking a peptide substrate (yellow) into the binding pocket¹⁵. A side view of the substrate domain is shown on the right. A cartoon depicting the two-domain complex is shown below. The bound nucleotide is shown in space filling format. **b** | In the ATP-bound state, the lid opens, and both the lid and the substrate-binding domain dock to the nucleotide-binding domain (PDB code: 4B9Q)²⁰. The corresponding cartoon of this conformation is shown below. When ATP binds, the cleft closes, triggering a change on the outside of the nucleotide-binding domain that creates a binding site for the linker region. Linker binding causes the substrate-binding domain and the lid domain to bind different sites on the nucleotide-binding domain, resulting in a widely opened substrate-binding site that enables rapid exchange of polypeptide substrates. After hydrolysis, the domains separate and the lid closes over the bound substrate. Such binding and release of extended regions of polypeptide chain are thought to unfold and stabilize non-native proteins either for correct folding or degradation.

HSP90 — a cellular signalling hub

HSP90, another highly abundant and ubiquitous chaperone, has diverse biological roles, but its mechanism of action is less well-understood than that of the major chaperones. It is a highly flexible, dynamic protein and, in eukaryotes, has a multitude of interactors that regulate its activities, making it a hub for many pathways^{31–34}. Like other stress proteins, HSP90 is capable of binding non-native polypeptides and preventing their aggregation. It seems to act mainly at the late stages of substrate folding. For example, steroid hormone receptors must bind HSP90 for efficient loading of their steroid ligand. The bacterial form seems to act alone and is not crucial for viability, but the eukaryotic forms and their many co-chaperones are essential. HSP90 is functionally more specialized than the other general chaperones. It is important for maturation of signalling proteins in development and cell division, and its substrates include steroid hormone receptors, kinases and key oncogenic proteins such as the tumour suppressor p53.

An intriguing evolutionary hypothesis proposes that HSP90 acts as a buffer for genetic variation by rescuing mutated proteins with altered properties³⁵. A reservoir

of such proteins could serve to improve fitness during evolutionary change. Some experimental support for this idea comes from studies investigating the developmental effects of HSP90 inhibitors on *Drosophila melanogaster* and *Arabidopsis thaliana*, and from studies examining the effects of environmental stress in yeast³⁶.

HSP90 in complex with nucleotides and substrates.

HSP90 forms a dimer of elongated subunits, with each subunit comprising three domains that are linked by flexible regions. It stably dimerizes through its carboxy-terminal domains and also transiently through its amino-terminal ATPase domain when ATP is bound³⁷ (FIG. 2). HSP90 is extremely dynamic, as it fluctuates rapidly between conformations ranging from an open V-shape to a closed form resembling a pair of cupped hands³⁸. The nucleotide-binding site accommodates a bent conformation of ATP, the binding of which causes transient dimerization of the N-terminal domains, characteristics of the GHKL (gyrase, HSP90, His kinase and MutL) ATPase fold shared with the DNA-unwinding enzyme DNA gyrase^{39,40}. Specific inhibitors

GHKL

An ATP-binding superfamily that includes DNA gyrase, the molecular chaperone heat shock protein 90, the DNA-mismatch-repair enzyme MutL and His kinase, which bind ATP in a characteristic bent conformation.

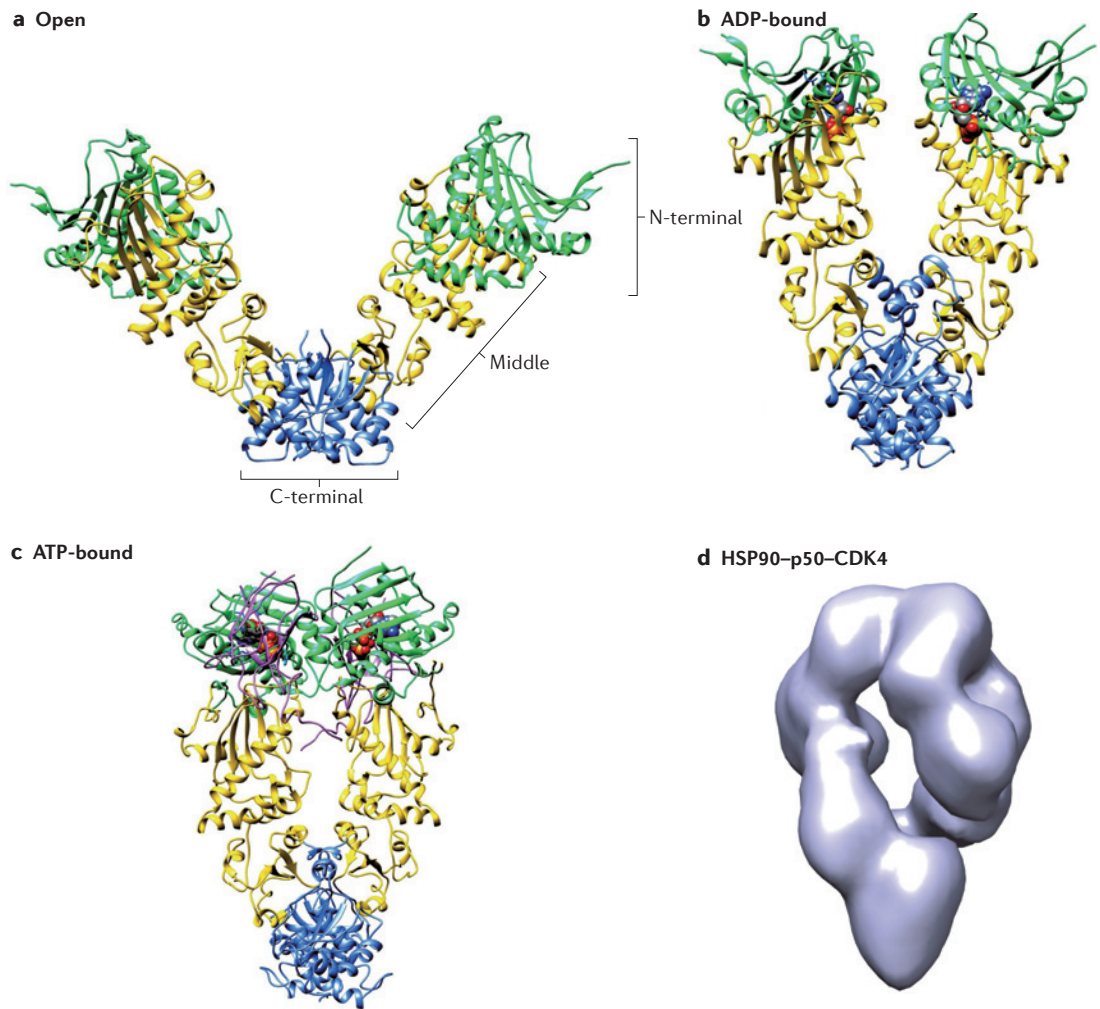


Figure 2 | HSP90 conformations and substrate binding. Crystal structures of heat shock protein 90 (HSP90) dimers in an open, unliganded state (Protein Data Bank (PDB) code: 2IQQ)¹²² (part **a**), a partly closed, ADP-bound state (PDB code: 2O1V)¹²³ (part **b**) and in a closed, ATP-bound state (PDB code: 2CG9)³⁷ (part **c**), are shown, and the amino-terminal domain (green), the middle domain (yellow) and the carboxy-terminal domain (blue) are indicated. The open form shown is *Escherichia coli* HptG, the partly closed ADP-bound form is the canine endoplasmic reticulum-associated HSP90 homologue GRP94 and the ATP-bound form (shown is the ATP analogue AMP-PNP) is yeast Hsc82 (heat shock cognate 82). Nucleotides are shown in space filling format. ATP favours binding to the closed form (part **c**), whereas hydrolysis or nucleotide release is favoured by a range of more open states (parts **a,b**). Opening and closing of the cleft are thought to mediate the action of HSP90 on its substrates, although the mechanisms underlying HSP90 action remain largely unclear. The electron microscopy map of HSP90 in complex with the cofactor p50 and its substrate cyclin-dependent kinase 4 (CDK4) is shown⁴⁸ (part **d**). Extra density of the side of this asymmetric complex is attributed to the cofactor and substrate.

of the HSP90 ATPase have marked effects in development and cancer. Although the HSP90 nucleotide state is only weakly coupled to its conformational change⁴¹, the many binding partners of HSP90 influence different steps in the functional cycle. HSP90 action is modulated by co-chaperones and client proteins (the term used for 'substrates' in the HSP90 system). In addition, phosphorylation, acetylation and other post-translational modifications affect its functional state³².

Co-chaperones that target HSP90 to specific types of client protein include p50 (also known as CDC37), which recruits kinases and inhibits the ATPase activity of HSP90 (REF. 42). A set of co-chaperones with prolyl isomerase activity, such as the immunophilin 52 kDa

FK506-binding protein (FKBP52), are involved in complexes with steroid receptors. These co-chaperones interact through their tetratricopeptide repeat (TPR) domains with a conserved C-terminal motif found on HSP90 and also on HSP70 (REF. 43). Numerous other co-chaperone complexes assemble on HSP90 via TPR domains. For example, HSC70–HSP90-organizing protein (HOP; also known as STI1) recruits HSP70 to HSP90, creating a complex for substrate handover⁴⁴. Important non-TPR containing co-chaperones include activator of HSP90 ATPase 1 (AHA1) and p23, which is involved in client protein maturation^{45,37}. Together with HSP70, HSP90 also has an important role in targeting substrates for degradation⁴⁶.

Details of substrate binding to HSP90 are poorly understood. Evidence for substrate-binding sites on all three domains of HSP90 came from low-resolution electron microscopy and mutational studies, which led to a model of hydrophobic surfaces lining the cavity of an open dimer^{47–50}. Despite the lack of a mechanistic understanding of the action of HSP90, specific inhibitors of its ATPase activity, such as geldanamycin, were shown to have important biological effects and form the basis for successful anticancer drugs⁵¹.

HSP60 — a protein folding container

Chaperonins (a term specific to this chaperone family) can be divided into two subfamilies: group I is composed of the bacterial chaperonin GroEL and its co-chaperonin GroES, as well as the mitochondrion- and chloroplast-specific HSP60 proteins together with their HSP10 co-chaperonins; and group II chaperonins, which are found in archaea and the eukaryotic cytosol and comprise the archaeal thermosome and eukaryotic CCT (chaperonin-containing TCP1; also known as TriC). In group II, an extra protein domain replaces the group I co-chaperonin. The bacterial GroEL–GroES chaperonin system is by far the best understood general chaperone.

Chaperonins are self-contained machines that leave little to chance; they provide a complete isolation chamber for protein folding. Early work on bacteriophage assembly, mitochondrial and chloroplast biogenesis led to the realization that related proteins in bacteria, chloroplasts and mitochondria have an essential role in *de novo* protein folding and assembly as well as refolding stress-denatured proteins^{52–54}.

Chaperonin structures and action. Biochemical, biophysical and structural analyses, particularly of *E. coli* GroEL–GroES, have revealed many important parts of the mechanism of action^{55,56}. GroEL crystal structures reveal details of the start and end states of extensive movements of this chaperonin through concerted rigid-body rotations of the subunit domains. Unliganded (apo) GroEL forms a 15 nm long cylindrical structure composed of back-to-back rings of seven 60 kDa subunits⁵⁷ (FIG. 3a). These rings surround open cavities of ~5 nm diameter, the walls of which are lined by a band of continuous hydrophobic surfaces. The two rings alternately go through cycles of ATP binding and hydrolysis. Upon ATP binding, a GroEL ring rapidly recruits the co-chaperonin GroES, a ring of seven 10 kDa subunits, which caps the cavity, entailing a dramatic structural reorganization to convert the open ring into an enclosed chamber with a hydrophilic lining⁵⁸. In the GroES-bound ring, the substrate-binding apical domains are elevated by 60° and twisted by 90° relative to the unliganded ring.

The open, hydrophobic lined ring is the acceptor state that captures non-native polypeptides with exposed hydrophobic surfaces, accounting for the lack of binding specificity of group I chaperonins. The interaction with the substrate can extend over 3–4 adjacent GroEL subunits^{59,60}. The actions of GroEL, ATP and GroES exert mechanical forces on the substrate that potentially result in unfolding of trapped, misfolded proteins⁶¹.

This culminates in a power stroke that ejects the substrate from the hydrophobic sites and simultaneously traps it inside the GroES-capped hydrophilic chamber for folding⁶². Once encapsulated, the lack of exposed hydrophobic sites or other partners for aggregation, together with the limited enclosure (~7 nm maximum dimension), blocks further misfolding or aggregation pathways, so that the substrate can either follow a folding pathway determined by its amino acid sequence or remain unfolded. After a slow ATP hydrolysis step, the chamber is re-opened, releasing the protein either committed to final folding and assembly or releasing it in a non-native state that will be recaptured by a chaperonin ring. For substrates that are too large to be encapsulated, GroES may still act allosterically to effect productive release of the substrate from the remote open ring⁶³.

Key to understanding this action is to determine the structures of the intermediate complexes when substrate and ATP have bound and GroES is being recruited. At low to intermediate resolution, substrate binding and GroEL domain movements have been characterized by single particle cryo-electron microscopy (cryo-EM) of various intermediate complexes, using statistical analysis to discriminate multiple three-dimensional structures from images of heterogeneous and dynamic complexes. This approach has yielded structural descriptions of chaperonin complexes at different stages of substrate binding and folding and has enabled analysis of the allosteric machinery^{60,64,65}.

Crystal structures as well as kinetic and mutational studies have revealed key allosteric sites in chaperonins. Each subunit contains three domains connected by flexible hinge points (FIG. 3b). The nucleotide-binding pocket is in the equatorial domain, and helix D runs from an Asp residue coordinating the γ -phosphate site to one of the two inter-ring contacts. In the GroES-bound state, the intermediate domain closes over the ATP pocket, bringing a catalytic Asp residue close to the nucleotide. Within each ring, the subunits are interlinked by salt bridges and act in concert, exhibiting positive cooperativity for ATP binding⁶⁶. Conversely, the two rings act sequentially, exhibiting negative cooperativity, which is transmitted through the two inter-ring contacts. Hydrophobic sites on the apical domain form the GroES- and substrate-binding sites (FIG. 3c). A mobile loop of GroES binds to the distal part of this site, a region also implicated in substrate binding, leading to the notion that GroES and substrate binding are mutually exclusive. However, there is biochemical evidence, although no direct structural information, for an intermediate state in which GroES and substrate are simultaneously bound to GroEL^{67,68}.

Substrate complexes. Crystal structures of extended peptides bound to the GroEL apical domain occupy the same site as the GroES mobile loop^{58,69,70}. This provides a partial view of how substrates might bind, but electron microscopy studies of GroEL with captured non-native proteins show a preference for binding deeper inside the cavity in the more proximal part of the hydrophobic site^{60,64}. Moreover, electron microscopy structures show how substrates bind to the open ring

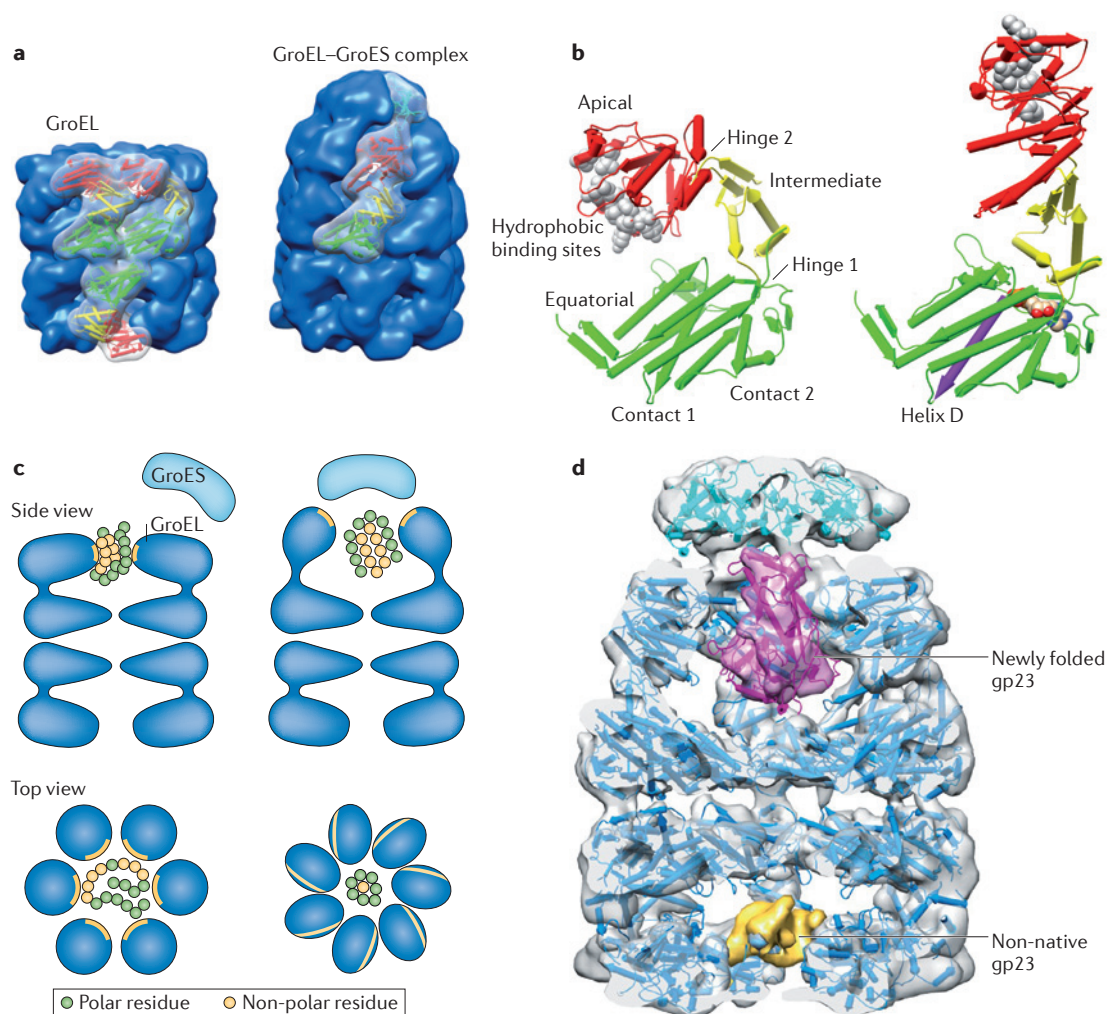


Figure 3 | GroEL conformations and substrate complexes. **a** | Overview of unliganded (apo) GroEL (Protein Data Bank (PDB) code:1OEL)⁵⁷ (left) and the GroEL–GroES complex (PDB code: 1SVT)⁵⁸ (right). The overall shapes are shown as blue surfaces, with three subunits coloured by domain in red, green and yellow in apo GroEL. One subunit of GroEL and one of GroES (cyan) are highlighted in the GroEL–GroES complex. **b** | Conformation of a GroEL subunit in the apo form (left) and the GroES-bound form (right), with GroEL key sites indicated (GroES is not shown). **c** | Cartoons of complexes with folding proteins. Hydrophobic surfaces and residues are shown in yellow and polar residues in green. **d** | Cut open view of the cryo-electron microscopy structure (Electron Microscopy Data Bank code: EMD-1548) of GroEL (PDB code: 1AON) in complex with bacteriophage 56 kDa capsid protein (gp31) (PDB code: 1G31), with a non-native gp23 (PDB code: 1YUE) bound to both rings⁶⁴. The pink density in the folding chamber corresponds to newly folded gp23, and the yellow density in the open ring is part of a non-native gp23 subunit. The corresponding atomic structures are shown embedded in the electron microscopy density map, except for the non-native substrate, which is unknown and only partially visualized owing to disorder. The open ring with its hydrophobic lining is the acceptor state for non-native polypeptides, and binding to multiple sites may facilitate unfolding. ATP and GroES binding to the chaperonin create a protected chamber with a hydrophilic lining that allows the encapsulated protein to fold.

and how they appear in the folding chamber (FIG. 3d). This enclosure imposes an upper limit of under 60 kDa for protein subunits that can be encapsulated. To accommodate its 56 kDa capsid protein, gp23, bacteriophage T4 encodes its own GroES homologue, gp31, to make the cage slightly taller⁷¹. The newly folded large domain of gp23, encapsulated and trapped by using a non-hydrolysable ATP analogue, fills the chamber and distorts it⁶⁴. A trapped, non-native state of another large substrate protein, RuBisCo (ribulose-1,5-bisphosphate carboxylase oxygenase), has been visualized by cryo-EM, revealing contacts to apical and equatorial domains⁷².

ATP complexes and domain movements. How does the binding of ATP detach a non-native protein multivalently bound on the hydrophobic surface, resulting in a free subunit isolated in the folding chamber? The conformation of open GroEL rings in the presence of ATP is extremely dynamic. Sorting of heterogeneous complexes by single particle electron microscopy has resolved a set of intermediate states that seem to be in equilibrium until a ring is captured by GroES⁶⁵. ATP binding causes small movements of the equatorial domains that are relayed both within and between rings. In the ATP-bound ring, the movements are amplified

into large rotations of the apical domains that culminate in dramatic reorganization of the substrate-binding surface. The movements involve a rotation about the equatorial–intermediate hinge, bringing the catalytic Asp residue near the nucleotide-binding pocket. This rotation leads to the breakage of two intersubunit salt bridges and transient generation of two new ones. In addition, the ATP-triggered domain movements are relayed through helix D (FIG. 3b) to the opposite ring via distortion of the inter-ring interface, thus mediating negative cooperativity⁷³.

The recently solved crystal structure of a GroEL double mutant lacking two key salt bridges reveals a remarkable, asymmetric ring with ADP bound to every subunit⁷⁴. The seven different subunit conformations correspond to those seen in the individual cryo-EM reconstructions⁶⁵. In the cryo-EM structures, the rings were observed to maintain sevenfold symmetry, except for the apical domains in the more open states.

The observed arrangements of the substrate-binding surface fall into four categories ([Supplementary information S1](#) (figure)). The distal part of the hydrophobic site is delineated by helix H and helix I. The collinear tracks of both helices lining the apo GroEL ring are distorted into tilted tracks with the end of helix H joined to the next helix I in one category of GroEL–ATP states. In these structures, the hydrophobic sites form a continuous band. In the more open GroEL–ATP states, the contacts between adjacent apical domains are completely lost, and the hydrophobic band becomes discontinuous. The free apical domains are not constrained to remain in symmetric positions. The open state has two important properties. First, radial expansion provides a plausible mechanism for forced unfolding of multivalently bound substrate. Second, combined with ring expansion, the elevation of the helix H–helix I groove creates a suitable docking site for the GroES mobile loops. In order to reach the folding-active, GroES-bound conformation, the GroES binding sites must each twist by 100° ([Supplementary information S2](#) (movie)). Thus, the open state is a good candidate for the initial GroES-docked intermediate: the mobile loops are highly flexible and can easily be modelled without the twist they adopt in the GroEL–GroES crystal structures. Moreover, the key parts of the substrate-binding site, helix I and the more proximal, underlying segment, are still exposed to the cavity. It has been proposed that a ternary complex between the open state GroEL–ATP, substrate and GroES represents the elusive intermediate, and that the 100° twist, triggered by binding of the GroES loops to produce the final bullet complex, would provide the power stroke that removes the hydrophobic binding site from the cavity and forcefully ejects the bound substrate into the chamber for folding⁶⁵.

Group II chaperonins. Group II chaperonins perform similar functions to group I chaperonins, and the underlying machinery is closely related. The most obvious structural difference between group I and group II chaperonins is the presence of a prominent insertion in the apical domain in group I chaperonins, which acts as

a substitute for GroES in capping the ring⁷⁵. The various archaeal forms usually have eightfold or ninefold symmetry, and eukaryotic CCT has eight related but distinct gene products forming the eight subunits of each ring. CCT in particular has been very difficult to study, and even the order of subunits in a ring is controversial^{76,77}. Unlike the archaeal forms and most other chaperones, CCT does not seem to be a HSP. CCT has specialized subunits, with some binding known substrates such as actin and tubulin. Various open, intermediate and closed conformations of intact group II complexes have been described by X-ray crystallography and cryo-EM (for example, REFS 78–81). An interesting difference in how the allosteric machinery operates is that the inter-ring interface is formed of 1:1 instead of 1:2 subunit contacts, leading to altered allosteric interactions^{82,83}. The unliganded form is open and dynamic, equivalent to the open state of GroEL. ATP analogue binding seems to gradually close the cage. Remarkably, although the apical domains undergo similar elevations and twists in group I and II chaperonins, these motions seem to occur in reversed sequence ([Supplementary information S3](#) (movie)). Overall, it seems likely that group II chaperonins perform similar actions as members of the group I family, but they exhibit different ATP-driven allosteric movements.

HSP100 disassembly machines

The HSP100 proteins are unfoldases and disaggregases, forceful unfolding motors that deliver substrates to compartmentalized proteases or disassemble aggregates containing misfolded proteins.

The AAA+ chaperones. HSP100 proteins are members of the AAA+ superfamily, which typically form oligomeric ring structures and have mechanical actions such as threading polypeptides or polynucleotides through a central channel in order to unfold or unwind them^{84,85}. AAA+ proteins function in various cellular processes, including the disassembly of complexes, for example the SNARE complexes that bring membranes together for vesicle fusion. The role of chaperone members of this family is best characterized in regulated proteolysis. At the core of these compartmentalized proteases is a stack of co-axial ATPase and protease rings, formed either by separate functional domains of a single subunit type (as in the bacterial Lon protease) or in separate ATPase and protease subunit rings (as in the HslUV (also known as ClpYQ) complex)⁸⁶ (FIG. 4a,b). In HslUV, both rings are hexameric, whereas others such as ClpAP have a symmetry mismatch with hexameric ClpA ATPase and heptameric ClpP protease rings⁸⁷. Although the eukaryotic proteasome is much more complex, it has the same core architecture, and its regulatory cap contains a heterohexameric of ATPase subunits (RPT1–RPT6) that performs the same unfolding and threading functions^{88,89}.

The defining feature of the superfamily is the AAA+ domain, which consists of an α - β subdomain and a smaller, helical subdomain⁸⁵ (FIG. 4c,d). The nucleotide-binding site is located at the subdomain interface.

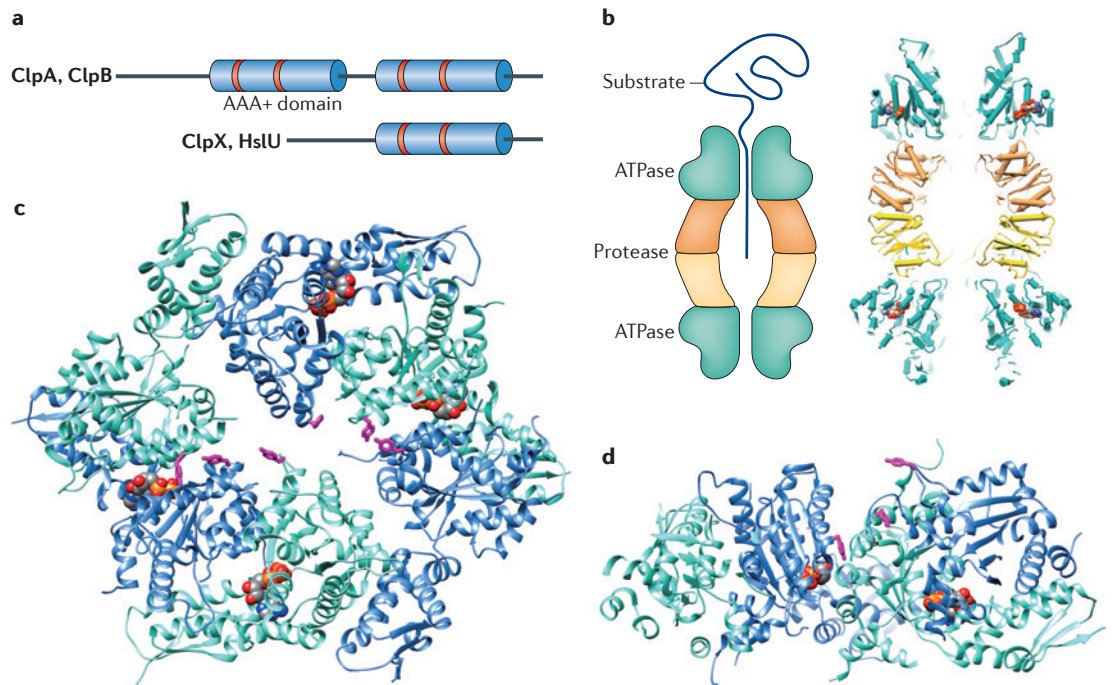


Figure 4 | HSP100 unfoldase. **a** | The two types of heat shock protein 100 (HSP100) sequences are shown schematically, with either a single or two tandem AAA+ domains. The characteristic Walker A and B sites are shown in red. **b** | The HslUV ATPase–protease complex is shown as a cartoon on the left, and the atomic structure is shown on the right (Protein Data Bank (PDB) code: 1G3I)¹²⁴. **c** | Top view of the asymmetric ClpX crystal structure (PDB code: 3HWS)⁹⁵. The four bound ADP molecules are shown in space-filling format and Tyr side chains on the pore loops are shown as magenta sticks. **d** | Side view section of ClpX showing the pore with three of the Tyr sites at different heights.

Conserved regions important in nucleotide binding and hydrolysis are the Walker A and Walker B motifs, sensor 1 and sensor 2 as well as the Arg finger involved in catalysis of the ATPase at the interface between subunits. AAA+ chaperones typically form hexameric rings that surround a narrow central pore lined with loops containing a substrate interaction site with aromatic and hydrophobic side chains. They exist as both single AAA+ rings (such as in HslU and ClpX) and stacked rings of tandem AAA+ domains (such as in ClpA, ClpB and ClpC). The HSP100 chaperones also have very mobile N-terminal domains that can play a part in substrate delivery to the central channel or interact with cofactors^{90,91}.

Unfolding during ATP-dependent proteolysis. How does unfolding work? First, the substrate is targeted to the entrance of the HSP100 channel. In bacteria, ribosome stalling causes expressed polypeptides to be marked for degradation by addition of an 11-residue peptide, the small, stable 10S RNA *ssrA* tag, which targets them to ClpXP or ClpAP⁹². Both ClpX and ClpA are powerful unfoldases that can even rapidly unfold a stable protein like GFP, if it is suitably tagged⁹³. The central channel is lined with Tyr residues on mobile pore loops that provide the binding sites for translocating chains, without specificity for sequence or chain polarity⁹⁴ (FIG. 4c,d). Once a polypeptide terminus or loop is engaged in the channel, rotations of the AAA+ subdomains, fuelled by the ATPase cycle, are thought to produce a rowing motion

to spool the unfolding chain through the channel. The structure of an asymmetric ClpX ring shows a sequence of pore loops at different heights in the channel and suggests a sequential or random action of the subunits around the ring⁹⁵ (FIG. 4d). Their axial separation of ~1 nm fits well with results obtained from single-molecule optical tweezer experiments showing translocation steps in multiples of 1 nm⁹⁶. Force and extension measurements support the action of a power stroke rather than a ratchet mechanism capturing random Brownian motions. The single-molecule approach shows that a C-terminal subdomain of GFP is extracted first, and this destabilizes the rest of the β -barrel, which unfolds before it is delivered to the surface of ClpX. Thus, for GFP, only the first unfolding step requires forceful pulling.

The AAA+ protein p97 (also known as CDC48 or VCP) functions in the transport of substrates to the proteasome, in particular of proteins that are misfolded in the ER and are retrotranslocated to the cytosol for degradation^{97,98}. p97 is a highly conserved protein with tandem AAA+ domains and a mobile N-terminal domain and has recently been suggested to represent the ancestral proteasome unfoldase ring⁹⁹. p97, together with cofactors, has various other roles when it is in close proximity to membranes. These functions relate more to the actions of family members such as NSF (*N*-ethylmaleimide-sensitive factor), which disassembles SNARE complexes at membrane surfaces after they have mediated vesicle fusion.

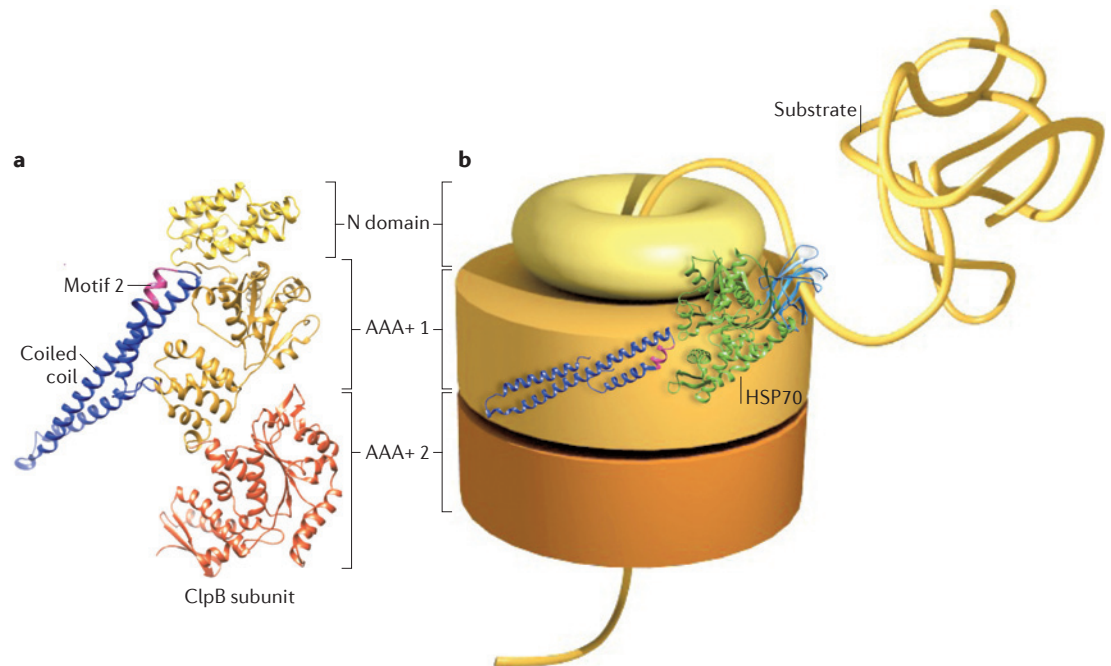


Figure 5 | **HSP100–HSP70 disaggregase.** The crystal structure of a ClpB subunit (Protein Data Bank (PDB) code: 1QVR)¹⁰³ (part **a**) and a schematic representation of the three-tiered hexamer are shown, with one ClpB coiled-coil domain (dark blue) bound to heat shock protein 70 (HSP70; with the nucleotide-binding domain shown in green and the substrate-binding domain in blue (PDB code: 4B9Q)) (part **b**). The ClpB–Hsp70 complex is derived from the model in REF. 106 combined with the structure of domain-docked HSP70 from REF. 20. The motif 2 sequence in the coiled-coil domain is highlighted in pink. A substrate polypeptide (yellow) is being extracted from an aggregate and threaded through the ClpB channel.

Protein disaggregation. A subset of the HSP100 chaperones found in bacteria, plants and fungi have the unique ability to reverse protein aggregation, in cooperation with their cognate HSP70 system^{100–102}. This subfamily includes *E. coli* ClpB and yeast Hsp104, which have tandem ATPase domains. A 90 Å long coiled-coil propeller, inserted near the end of the first ATPase domain¹⁰³, couples their unfolding and translocation actions to HSP70 (REF. 104) (FIG. 5a). Binding of the HSP70 ATPase domain to one end of the coiled-coil, a region highly sensitive to mutations and known as motif 2, is required for disaggregation^{105,106}. Docking to low-resolution and symmetrized electron microscopy maps yielded controversial results regarding the hexamer arrangement and the degree of expansion of the ring. A model based on studies of *Thermus thermophilus* ClpB proposes that the subunits are tightly packed around a 15 Å channel and the coiled-coils protrude as radial spikes¹⁰³. By contrast, electron microscopy maps of yeast Hsp104 suggest a much more expanded ring with a wide channel and the coiled-coils intercalated between the subunits, partly buried and partly exposed on the surface, with the HSP70-binding tip of the coil adjacent to the N-terminal ring¹⁰⁷. More recent cryo-EM maps of HSP104 are interpreted as typical AAA+ rings with the coiled-coils on the outside, but no density is observed for the coiled-coils¹⁰⁸. A low-resolution crystal structure of the hexameric assembly of ClpC, a protease-coupled HSP100 with tandem AAA+ domains and a partial coiled-coil structure, shows an expanded ring and the coiled-coil lying

tangentially on the surface¹⁰⁹. However, ClpC lacks the HSP70-binding arm of the coil and requires the cofactor MecA for hexamer assembly. Recent work probing accessibility and hydrogen–deuterium exchange on the coiled-coil domain of *E. coli* ClpB does not support either model. Rather, it suggests that the coil lies on the surface of the hexamer, with motif 2 being protected when ClpB activity is repressed and being accessible when ClpB is active¹¹⁰ (FIG. 5b).

Methyl TROSY NMR has recently been used to model the local interactions between the ClpB coiled-coil and the DnaK ATPase domain in its open, ADP-bound state¹⁰⁶. Combining this model with the model of domain-docked DnaK suggests how DnaK might deliver a polypeptide segment to ClpB (FIG. 5b). This speculative, combined model suggests that the ClpB coiled-coil adopts a more vertical orientation to bring the DnaK substrate-binding domain to the vicinity of the pore channel. The N-terminal domains of ClpB might play a part in delivering the substrate from DnaK to ClpB, after DnaJ makes initial weak contact with the surface of the aggregate and hands over a segment of the polypeptide for engagement with DnaK.

Conclusions

Chaperones are nanoscale molecular machines that recognize incompletely or incorrectly folded proteins, arrest or unfold them and then either release them for spontaneous refolding or target them for degradation. With the help of many cofactors, the general purpose chaperone

HSP70, a two-domain monomer, carries out all these actions. Another ‘sociable’ chaperone, HSP90, acts as a flexible dimer, with even more partners to regulate its activities. In a more solitary action, the HSP60 chaperonins assist folding by creating an isolation chamber for the substrate protein. Most forceful of all, the HSP100 protein remodellers can rip apart even stably folded proteins or disassemble large and otherwise irreversible aggregates.

HSP70 and HSP90 have many surface exposed interaction sites for cofactors, giving them a high degree of regulation and integration into other cellular pathways. By contrast, the HSP60 and HSP100 families are largely inward looking, and they enclose their active sites with few cofactors. Their activities are mainly regulated by a stress-induced increase in their expression levels. A striking feature of the ATPase cycles of these chaperones is their highly dynamic nature. Rather than simple conformational switching, the massive domain movements in chaperone action are only loosely coupled to their nucleotide-bound state. Nevertheless, each of these chaperone families has a distinct mode of ATP binding, ranging from the unique chaperonin nucleotide site to the very widespread Walker A and Walker B type

ATPase in HSP100. HSP70 shares its nucleotide-binding fold with actin and hexokinase, whereas HSP90 has a GHKL nucleotide-binding fold characteristic of DNA gyrase. The nucleotide binds in an extended conformation to HSP60 and HSP70 but is bent when bound to HSP90 and HSP100, giving rise to different specificities for nucleotide analogues ([Supplementary information S4](#) (figure)).

Although the chaperone systems discussed here have a fairly broad range of substrates, many proteins have specific requirements for chaperones and co-chaperones. For example, the substrates of group I and group II chaperonins are quite distinct; specific HSP40 co-chaperones are required together with HSP70 for the folding of many important substrates. The mechanisms of this specificity are poorly understood. A major current question is why the chaperone systems become less effective in ageing organisms, leading to the eventual failure of protein quality control and the onset of misfolding diseases. Future progress in the field will require high-resolution structures of chaperone complexes acting on misfolded or unfolded proteins, the identification of specific causal pathways in aggregate and amyloid toxicity, as well as a better understanding of the regulation of proteostasis.

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The author declares no competing financial interests.

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