Molecular Chaperone Functions in Protein Folding and Proteostasis

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Abstract

The biological functions of proteins are governed by their three-dimensional fold. Protein folding, maintenance of proteome integrity, and protein homeostasis (proteostasis) critically depend on a complex network of molecular chaperones. Disruption of proteostasis is implicated in aging and the pathogenesis of numerous degenerative diseases. In the cytosol, different classes of molecular chaperones cooperate in evolutionarily conserved folding pathways. Nascent polypeptides interact cotranslationally with a first set of chaperones, including trigger factor and the Hsp70 system, which prevent premature (mis)folding. Folding occurs upon controlled release of newly synthesized proteins from these factors or after transfer to downstream chaperones such as the chaperonins. Chaperonins are large, cylindrical complexes that provide a central compartment for a single protein chain to fold unimpaired by aggregation. This review focuses on recent advances in understanding the mechanisms of chaperone action in promoting and regulating protein folding and on the pathological consequences of protein misfolding and aggregation.

Contents

Molecular chaperone: any protein that interacts with and aids in the folding or assembly of another protein without being part of its final structure

Proteostasis: the state of balanced proteome found in healthy cells

Amyloid: disease-associated, fibrillar aggregates with cross-β structure

INTRODUCTION

The successful execution of cellular processes depends on the coordinated interactions of proteins. In humans, an estimated 20,000 to 25,000 different proteins are responsible for most aspects of biological function. Following synthesis on ribosomes as linear sequences of amino acids, the vast majority of proteins must fold into well-defined three-dimensional structures (their native states) to attain functionality. Although some newly translated proteins are able to fold spontaneously, a substantial fraction of proteins are less efficient at folding and vulnerable to misfolding, a problem exacerbated by the highly crowded cellular environment (1). In particular, large proteins with complex structures may expose hydrophobic amino acid residues to the solvent during folding, rendering them susceptible to nonnative (off-pathway) interactions that lead to aggregation (2, 3). To counteract these nonnative interactions, cells have a network of molecular chaperones that assist in de novo folding and maintain preexisting proteins in their native states (4–7). A key role of molecular chaperones is preventing protein aggregation, especially under conditions of cellular stress. Moreover, the molecular chaperone network functions in diverse aspects of protein quality control, including protein unfolding and disaggregation and targeting terminally misfolded proteins for proteolytic degradation.

Imbalances in protein homeostasis (proteostasis) are observed in an increasing number of disease states, emphasizing the importance of cellular protein quality control (8). The predominant feature of these disorders is protein misfolding as manifested by the formation of intracellular and/or extracellular deposits of aggregated proteins. Examples include the formation of intracellular inclusions containing aggregated α-synuclein in Parkinson's disease or huntingtin in Huntington's disease, as well as the extracellular β-amyloid plaques in Alzheimer's disease (9). Deficiencies in proteostasis are also observed in many other age-related diseases, such as type II diabetes, peripheral amyloidosis, cancer, and cardiovascular diseases. Indeed, studies using model organisms demonstrate that a gradual decline in cellular proteostasis capacity occurs with aging (10).

Here, we review recent advances in understanding the role of molecular chaperones in protein folding and proteostasis maintenance. We focus our discussion on the cytosolic chaperone networks and the pathological consequences of their disruption. For a discussion of protein folding in the secretory pathway and the folding of membrane proteins, we refer the reader to recent reviews (11, 12, 13).

Protein Folding and Aggregation

The folded three-dimensional structures of most proteins represent a compromise between

thermodynamic stability and the conformational flexibility required for function. Consequently, proteins are often marginally stable in their physiological environment and thus susceptible to misfolding and aggregation (2, 3). In addition, a substantial fraction of proteins in eukaryotic cells (~30%) are classified as intrinsically unstructured and contain regions thought to adopt ordered structure only upon interaction with binding partners (14). Such proteins may be metastable and prone to aggregation.

The pioneering studies of Anfinsen revealed that small denatured proteins refold spontaneously in vitro, demonstrating that the three-dimensional structure of a protein is encoded in its amino acid sequence (2, 3). Much progress in recent years has helped us understand how exactly the linear sequence of amino acids encodes the native state of a protein and directs its folding process. Because the number of possible conformations a protein chain can adopt is very large, folding reactions are highly complex and heterogeneous, relying on the cooperation of multiple weak, noncovalent interactions. Among these, hydrophobic forces are critical in driving chain collapse and the burial of nonpolar amino acid residues within the interior of the folding protein. Polypeptide chains are thought to explore funnel-shaped potential energy surfaces as they progress toward the native structure along several downhill paths rather than a single defined pathway (**Figure 1**) (2). Rapid chain collapse and the incremental formation of native contacts limit the conformational space that must be searched en route to the native state. However, the rugged free-energy surface navigated during folding often requires molecules to cross substantial kinetic barriers. As a result, kinetically trapped folding intermediates and misfolded states may be transiently populated. Misfolded states are characterized by the presence of nonnative interactions that must be resolved prior to correct folding. Productive folding intermediates may display a high degree of configurational flexibility, increasing the search time required for the formation of native intrachain contacts. The propensity of proteins to populate such entropically stabilized intermediates increases with larger, more topologically complex domain folds that are stabilized by many long-range interactions (such as α/β domain architectures). Such proteins are often highly chaperone dependent (15).

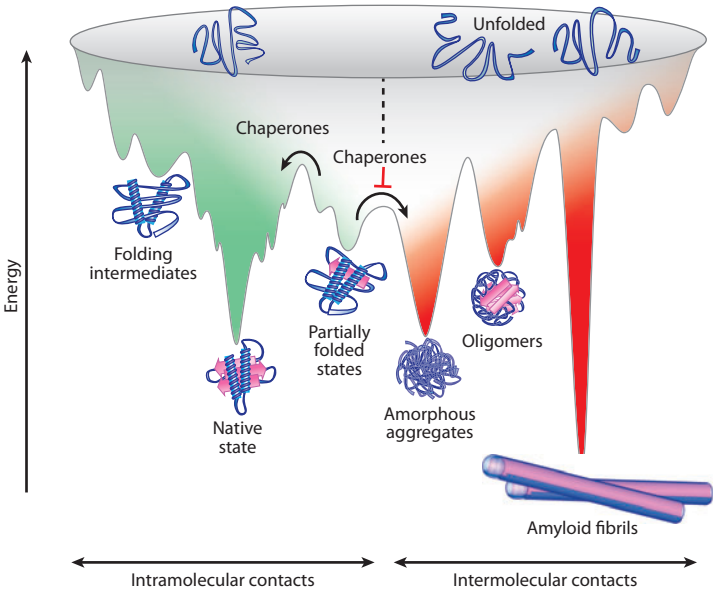


Figure 1

Competing reactions of protein folding and aggregation. Proteins fold by sampling various conformations in a folding energy landscape. Energetically favorable intramolecular interactions (*green*) are stabilized as the protein progresses on a downhill path through the landscape toward the native state. Energetically favorable but nonnative conformations result in populations of kinetically trapped states that occupy low-energy wells (partially folded states or misfolded states). Chaperones assist these states in overcoming free energy barriers and prevent intermolecular interactions (*red*) leading to aggregation (amorphous aggregates, β-sheet-rich oligomers, and amyloid fibrils), thus promoting folding to the native state. Figure adapted and modified from References 17 and 237.

Partially folded or misfolded states typically expose hydrophobic amino acid residues and regions of unstructured polypeptide backbone to the solvent, the features that mediate aggregation in a concentration-dependent manner (**Figure 1**) (9, 16). Although aggregation primarily leads to amorphous structures largely driven by hydrophobic forces, it may also lead to the formation of amyloid-like fibrils (**Figure 1**). These fibrillar aggregates are characterized by β-strands that run perpendicular to the long

Protein folding: the process by which the extended, newly synthesized polypeptide chain collapses into its functional three-dimensional conformation

fibril axis (cross-β structure) and are associated with diseases of aberrant protein folding. Many proteins can adopt these highly ordered, thermodynamically stable structures in vitro, but molecular chaperones restrict their formation in vivo (9, 16, 17). The formation of soluble oligomeric intermediates often accompanies the formation of fibrillar aggregates (**Figure 1**). The pronounced toxicity of these less-ordered and rather heterogeneous forms likely correlates with the exposure of interaction-prone hydrophobic surfaces, which in the fibril are integrated into a compact cross-β core (18). We can view certain oligomers as kinetically trapped intermediates that must undergo considerable structural rearrangement to form fibrils, the thermodynamic end state of aggregation (**Figure 1**). The relative impact of fibrillar and oligomeric aggregates on proteostasis and cell health is currently under intense investigation.

Protein Folding In Vivo

Attempts to understand protein folding in vivo must take into consideration the dramatic differences in physical properties that exist between the cellular environment and the conditions of test-tube refolding (19, 20). Compared with the dilute conditions in vitro, the cellular environment is highly crowded, containing concentrations of 300–400 mg ml^{-1} of protein and other macromolecules (1, 21). Macromolecular crowding results in excluded volume effects, limiting the entropic freedom of the folding polypeptide chains and favoring compact nonnative states (1, 22). In addition, macromolecular crowding enhances protein aggregation (amorphous and fibrillar) by increasing the affinities between interacting macromolecules including folding intermediates (1).

Protein folding in vivo is further complicated by its coupling with translation and by the fact that many newly synthesized polypeptides must be transported into subcellular compartments, such as the endoplasmic reticulum (ER) or the mitochondria (23), prior to folding. The vectorial translation process from the N terminus to C terminus places considerable restrictions on the energy landscape of in vivo folding (24, 25). The exit tunnel of the large ribosomal subunit, ~100 Å long and ~20 Å wide, precludes folding beyond the formation of α-helices or small tertiary structural elements that may begin to form near the tunnel exit (26–29). Thus, the C-terminal 30–40 amino acid residues of the nascent chain cannot participate in the long-range interactions necessary for cooperative domain folding. Consequently, productive folding is delayed until a complete protein domain (~50–300 amino acid residues), or substantial segments thereof, has emerged from the ribosome (30–35). Whereas single-domain proteins complete folding posttranslationally (after chain termination and release from the ribosome), proteins consisting of multiple domains may fold cotranslationally as the domains emerge sequentially from the ribosome. Sequential folding of the domains prevents the formation of unproductive intermediates resulting from nonnative interactions between concomitantly folding domains (36, 37). For the multidomain protein firefly luciferase, sequential domain folding results in a dramatic acceleration of folding speed (6, 7, 36). The slower translation speed in eukaryotes (~4 amino acids s^{-1}) compared with bacteria (~20 amino acids s^{-1}) (38), together with evolutionary adaptations of the chaperone machinery, may facilitate cotranslational folding for domains with slower folding kinetics and thus may have contributed to the explosive evolution of multidomain proteins in eukaryotes. Although domain size was conserved during evolution, the average size of proteins increased from ~35 kDa in bacteria such as *Escherichia coli* to ~52 kDa in humans (36). Translational pausing may also enhance the efficiency of cotranslation folding, but the significance of this phenomenon in vivo is still under investigation (34, 39–42).

The fastest translation speeds are slow compared with the rapid kinetics of folding observed for small protein domains in vitro, some of which fold on the microsecond to millisecond timescale (2, 3). A nascent chain

of average length (~300 amino acid residues in *E. coli*) will be exposed on the ribosome in an unfolded state for ~15 s, during which it may engage in nonnative intra- and interchain interactions. Contrary to the previous belief that polysomes enhance aggregation by increasing the local concentration of nascent chains, recent studies suggest that the three-dimensional organization of individual ribosomes in polysomes maximizes the distance between nascent chains, thus reducing the probability of unproductive interactions (43).

THE MOLECULAR CHAPERONE CONCEPT

We define a molecular chaperone as any protein that interacts with and aids in the folding or assembly of another protein without being part of its final structure (4). Chaperones are classified into different groups on the basis of sequence homology. Many are stress proteins or heat shock proteins (Hsps), as their synthesis is induced under conditions of stress (e.g., heat shock or oxidative stress), which structurally destabilize a subset of cellular proteins. Members of the various groups of chaperones were initially named according to their molecular weight: Hsp40s, Hsp60s, Hsp70s, Hsp90s, Hsp100s, and the small Hsps. Besides their fundamental role in de novo protein folding, chaperones are involved in various aspects of proteome maintenance, including assistance in macromolecular complex assembly, protein transport and degradation, and aggregate dissociation and refolding of stress-denatured proteins.

Chaperone-Mediated Folding by Kinetic Partitioning

Chaperones that function broadly in de novo folding and refolding (i.e., the chaperonins, Hsp70s, and Hsp90s) are ATP regulated and recognize segments of exposed hydrophobic amino acid residues, which are later buried in the interior of the natively folded protein. Binding to hydrophobic segments enables these chaperones to recognize the nonnative states of many different proteins. Folding is then promoted during ATP- and cochaperone-regulated cycles of binding and release of nonnative protein (**Figure 2**). In this mechanism of kinetic partitioning, (re)binding to chaperones blocks aggregation and reduces

Assembly: the association of two or more protein molecules in a functional complex

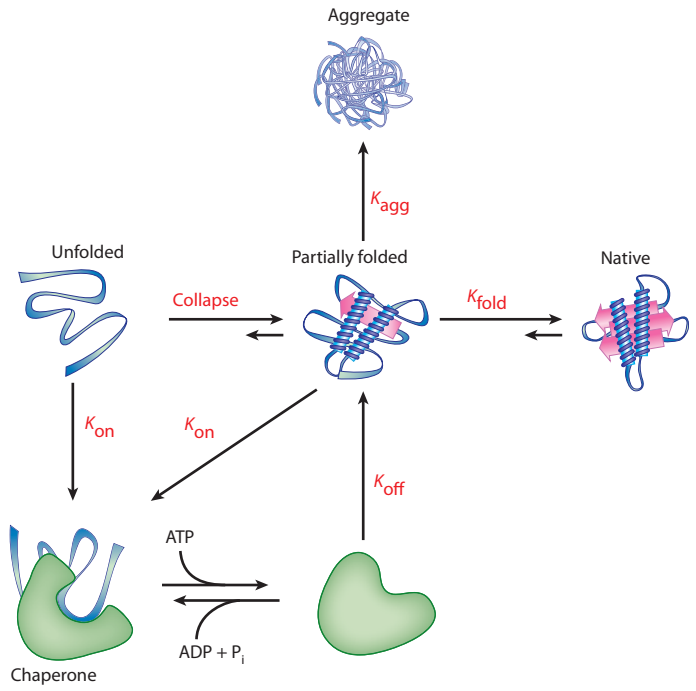


Figure 2

Molecular chaperones promote protein folding through kinetic partitioning. Under physiological conditions, the unfolded state of a protein undergoes rapid collapse to a partially folded, compact intermediate. Although chain collapse restricts the conformational space that must be searched en route to the native state, the collapsed folding intermediates are often aggregation-prone, kinetically trapped states. Many molecular chaperones switch between high- and low-affinity states for unfolded and partially folded proteins in a manner regulated by ATP binding and hydrolysis. Folding proceeds efficiently when the folding rate constant (K_{fold}) is greater than the rate constants for chaperone binding (K_{on}) and aggregation (K_{agg}). Binding or rebinding of nonnative protein to chaperone allows kinetic partitioning, preventing aggregation and favoring folding. When K_{on} is greater than K_{fold}, the chaperone system functions as a holdase, stabilizing the protein in a nonaggregated state for transfer to other chaperone systems or for degradation. During exposure to conformational stress, K_{agg} becomes greater than K_{on}, resulting in aggregation unless the cell upregulates its chaperone capacity through stress response pathways. K_{off} is the rate constant for protein release from chaperone and P_i is inorganic phosphate. Figure adapted and modified from Reference 38.

Stress proteins: cellular proteins whose expression is induced or increased under conditions of conformational stress; not all molecular chaperones are stress proteins

Aggregate: the association of two or more protein molecules in a nonfunctional state

Chaperonin: a class of structurally related molecular chaperones forming large, double-ring complexes that transiently enclose substrate protein for folding

TF: trigger factor

NAC: nascent-chain-associated complex

Nascent polypeptide: the polypeptide chain emerging from the ribosome during translation

RAC: ribosome-associated complex

the concentration of free folding intermediates, whereas transient release of bound hydrophobic regions is necessary for folding to proceed (**Figure 2**). ATP-independent chaperones, such as the small Hsps, may function as additional holdases that buffer aggregation. Efficient folding is achieved when the rate of folding is faster than the rates of aggregation or chaperone rebinding. If folding is slower, then the protein may be transferred to a chaperone system with different mechanistic properties, as exemplified by the sequential cooperation between Hsp70 chaperones and the cylindrical chaperonins (discussed below). Alternatively, transfer to the degradation machinery may occur. Aggregation occurs if the concentration of folding intermediates exceeds the available chaperone capacity. Such a situation generally results in the induction of the cellular stress response, which increases the abundance of stress-regulated chaperones.

Role in Protein Evolution

The general function of chaperones in assisting protein folding is significant in facilitating the structural evolution of proteins. By maintaining nonnative proteins in a soluble, folding-competent state, chaperones are thought to buffer mutations in proteins that would otherwise preclude their folding, thus broadening the range of mutant proteins subject to Darwinian selection (44, 45). After selection of a mutant protein with favorable functional properties, secondary mutations may improve its folding efficiency and solubility, allowing the protein to become less chaperone dependent and increase in abundance. Interestingly, proteins that depend highly on a specific chaperone system, such as the *E. coli* chaperonin GroEL, are of less than average abundance and often have nonessential functions (15). Conversely, highly abundant proteins with essential functions tend to be less dependent on a specific chaperone and may use multiple chaperone systems to optimize folding yield (46). Notable exceptions include the photosynthetic enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) as well as the cytoskeletal proteins actin and tubulin. Although highly abundant, these proteins are obligate substrates of the chaperonin system for folding (4, 6). Presumably, RuBisCO, actin, and tubulin reside in an evolutionarily trapped state in which further mutations that reduce chaperonin dependence are incompatible with function.

CHAPERONE PATHWAYS IN THE CYTOSOL

The general organization of cytosolic chaperone pathways is highly conserved throughout evolution (**Figure 3**). In all domains of life (bacteria, archaea, and eukarya), ribosome-binding chaperones [e.g., trigger factor (TF), nascent-chain-associated complex (NAC), and specialized Hsp70s] interact first with the nascent polypeptide, followed by a second tier of chaperones without direct affinity for the ribosome (the classical Hsp70 system). Folding may begin cotranslationally and finish posttranslationally upon chain release from the ribosome or after transfer to downstream chaperones (e.g., the chaperonins and Hsp90 system) (**Figure 3**). Recent system-wide and bioinformatic approaches identified the substrate interactome of several major chaperones, revealing the cooperative organization of the chaperone network (15, 47–57).

Chaperone Action on the Ribosome

As discussed above, the nascent polypeptide chain is topologically restricted until a complete protein domain is synthesized and emerges from the ribosomal tunnel. Ribosome-binding chaperones prevent emerging nascent chains from engaging in unfavorable intra- and intermolecular interactions during translation, typically by shielding exposed hydrophobic segments. The ribosome-associated molecular chaperones include TF (in prokaryotes) and specialized Hsp70 complexes such as ribosome-associated complex (RAC; in *Saccharomyces cerevisiae*), MPP11 and Hsp70L1

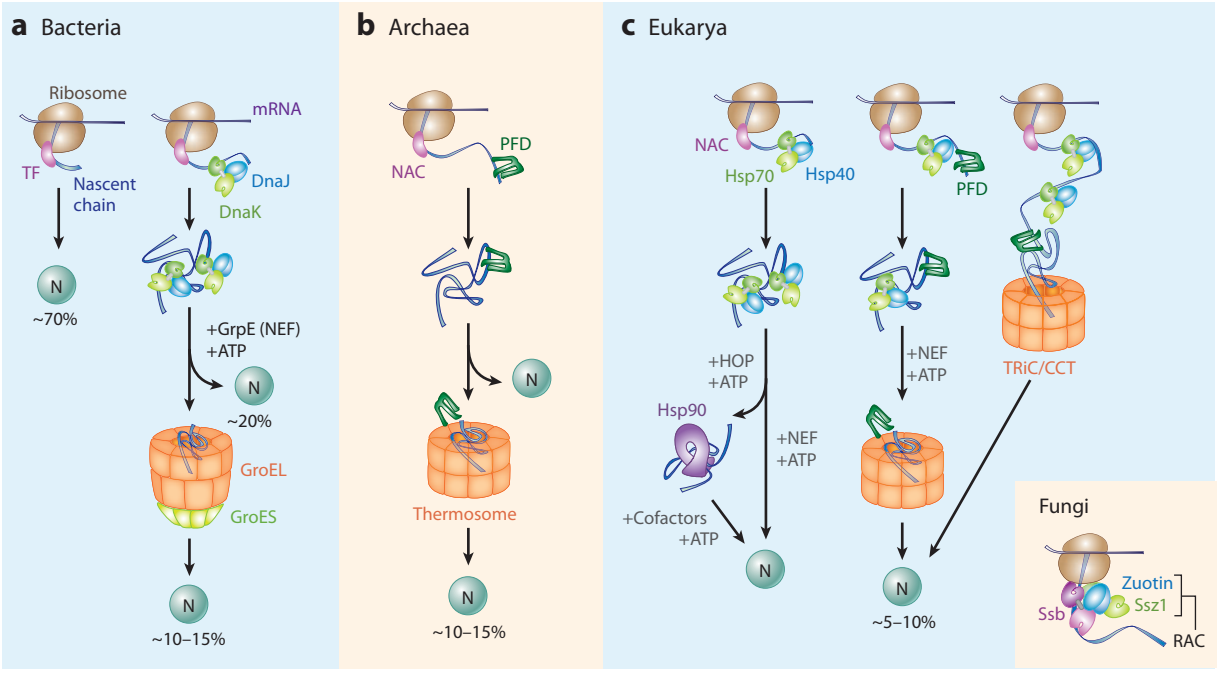


Figure 3

Organization of chaperone pathways in the cytosol. In bacteria (*a*), archaea (*b*), and eukarya (*c*), ribosome-bound chaperones [trigger factor (TF) in bacteria, nascent-chain-associated complex (NAC) in archaea and eukarya] aid folding cotranslationally by binding to hydrophobic segments on the emerging nascent chains. For longer nascent chains, members of the heat shock protein (Hsp)70 family (DnaK in bacteria and Hsp70 in eukarya), together with Hsp40s and nucleotide exchange factors (NEFs), mediate co- and posttranslational folding. In archaea lacking the Hsp70 system, prefoldin (PFD) assists in folding downstream of NAC. Partially folded substrates may be transferred to the chaperonins [GroEL-GroES in bacteria, thermosome in archaea, and tailless complex polypeptide-1 (TCP-1) ring complex (TRiC)/chaperonin-containing TCP-1 (CCT) in eukarya]. The Hsp90 system also receives its substrates from heat shock cognate 70 (Hsc70) and mediates their folding with additional cofactors. The insert in panel *c* shows the ribosome-binding chaperone system, the ribosome-associated complex (RAC), in fungi. RAC consists of Ssz1 (a specialized Hsp70) and zuotin (Hsp40) and assists nascent chain folding together with another Hsp70 isoform, Ssb. Percentages indicate the approximate protein flux through the various chaperones. Figure adapted and modified from Reference 7.

(in mammals), and NAC (in archaea and eukaryotes) (**Figure 3**) (17, 58, 59).

TF binds to the large ribosomal subunit at the opening of the ribosomal exit tunnel (60–62) and interacts with most newly synthesized cytosolic proteins as well as a subset of secretory proteins (55, 56, 58, 59). In vitro, TF binds to nascent chains as short as ~60 amino acid residues, presumably when the first hydrophobic segment of the chain has emerged (58, 63). In vivo, TF preferentially binds ribosomes when nascent chains have reached ~100 amino acids in length (55), thereby permitting prior interactions of ribosome-binding targeting factors (e.g., signal recognition particle) (64, 65) and modifying enzymes (e.g., peptide deformylase) (66) with the nascent chains. Release of TF from the nascent chain is ATP independent and permits folding or polypeptide transfer to downstream chaperones such as DnaK, the major Hsp70 in bacteria. Although the combined deletion of the genes encoding TF and DnaK is lethal at temperatures above 30°C, *E. coli* cells tolerate individual deletions of TF or DnaK, indicating that these proteins are functionally redundant (56, 58, 67).

In eukaryotes, RAC and NAC may fulfill a role similar to TF, although they are not structurally related to TF. RAC comprises the Hsp70-like protein Ssz1 (also known as Pdr13)

NEF: nucleotide exchange factor

TRiC: tailless complex polypeptide-1 ring complex

and the Hsp70 cochaperone zuotin (Hsp40) (**Figure 3*c***) (58, 59, 68–71). In fungi, RAC cooperates with ribosome-binding isoforms of Hsp70, Ssb1, or Ssb2. NAC, a heterodimeric complex of α- (33 kDa) and β- (22 kDa) subunits, also associates with ribosomes (via the β-subunit) and short nascent chains (54, 59, 72, 73). The exact role of NAC in folding or protein quality control is not established. In yeast, NAC function appears to be partially redundant with that of Ssb (71), reminiscent of the interplay between TF and DnaK in bacteria. Both Ssb-RAC and NAC may have a role in ribosome biogenesis (71). Archaea have a homolog of α-NAC.

Chaperones Acting Downstream of the Ribosome

In bacteria and eukaryotic cells, the classical Hsp70s have a central role in the cytosolic chaperone network (6, 17). They interact with a multitude of nascent and newly synthesized polypeptides but have no direct affinity for the ribosome (56, 74). The nascent-chain-interacting Hsp70 chaperones include DnaK in bacteria and some archaea, Ssa1–4 in yeast, and the constitutively expressed heat shock cognate 70 (Hsc70) in metazoan and mammalian cells (4, 58). Hsp70 chaperones function with cochaperones of the Hsp40 family (also known as DnaJ proteins or J proteins) and nucleotide exchange factors (NEFs) (**Figure 3**) (75). In addition to protecting nascent chains against aberrant interactions, the Hsp70-Hsp40 chaperone systems assist folding co- or post-translationally through ATP-regulated cycles of substrate binding and release (by kinetic partitioning; **Figure 2**) and mediate polypeptide chain transfer to downstream chaperones. Remarkably, most species of archaea lack the Hsp70 chaperone system. The chaperone prefoldin (also known as the Gim complex, GimC) may substitute for Hsp70 in these cases (7). Prefoldin, a hexamer of ~14–23 kDa α- and β-subunits with long α-helical coiled coils, binds in an ATP-independent manner to certain nascent chains and mediates their transfer to the cylindrical chaperonin complex for the final stages of folding. In eukaryotes, prefoldin participates in the chaperonin-assisted folding of actin and tubulin (6, 7).

Proteins that are unable to utilize Hsp70 for folding are transferred to the chaperonin or the Hsp90 system. Chaperonins (also referred to as Hsp60s) are large double-ring complexes of 800–1,000 kDa with a central cavity, which permits protein molecules to fold in an isolated compartment protected from the aggregation-promoting cytosol (**Figure 3**) (4, 5). The chaperonins are structurally classified into group I and group II (76, 77). Group I chaperonins include GroEL in bacteria, Hsp60 in mitochondria, and Cpn60 in chloroplasts. They cooperate with lid-shaped cochaperones (GroES, Hsp10, Cpn10/20) to encapsulate substrates. The group II chaperonins include the archaeal thermosome and its eukaryotic homolog tailless complex polypeptide-1 (TCP-1) ring complex (TRiC), also known as chaperonin-containing TCP-1 (CCT), which have a built-in lid. GroEL-GroES assist folding posttranslationally, whereas TRiC may assist folding co- and posttranslationally (6). TRiC binds to nascent chains and cooperates with Hsp70 in the cotranslational folding of multidomain proteins (78). Investigators have demonstrated a direct interaction between Hsp70 and TRiC (79). The chaperonins interact with 10–15% of newly synthesized polypeptides in bacteria and archaea (15, 52, 53) and 5–10% in eukarya (50). The obligate substrates of GroEL typically include proteins with complex domain folds, which tend to populate kinetically trapped folding intermediates (15, 53). Established TRiC substrates include the cytoskeletal proteins actin and tubulin as well as several proteins with β-propellers/WD40 repeats (49, 50).

In the eukaryotes, many signaling proteins are transferred from Hsp70 to the ATP-dependent Hsp90 chaperone system for completion of folding and conformational regulation (**Figure 3*c***) (45, 48, 80). Examples of Hsp90-mediated conformational regulation include nuclear hormone receptors, which Hsp90 stabilizes in a conformation poised for hormone binding and activation. Substrate

transfer to Hsp90 is mediated by the Hsp90 organizing protein (HOP; also known as Sti1 and p60), which uses multiple tetratricopeptide repeat (TPR) domains to bridge Hsp70 and Hsp90 (81). Various cochaperones facilitate the interaction of Hsp90 with steroid hormone receptors and multiple protein kinases (45, 48). Accordingly, Hsp90 affects many key cellular processes, including cell cycle progression, steroid signaling, calcium signaling, protein trafficking, protein secretion, the immune response, and the heat shock response (HSR) (45, 48, 82). Pharmacologic inhibition of Hsp90 with geldanamycin and derivatives results in the downregulation of many kinases (83) and is in clinical development for cancer therapy (84).

The molecular chaperone network is central to cellular protein quality control through its involvement in the conformational maintenance of proteins, the dissociation of aggregates, and the degradation of misfolded proteins. In yeast and other fungi, the cytosolic Hsp70 system cooperates with the AAA+ (ATPases associated with various cellular activities) chaperone Hsp104 in dissociating and refolding aggregated proteins. Hsp104 is homologous to bacterial ClpB, which functions with DnaK in protein disaggregation (85). Various cochaperones of Hsp70 and Hsp90 escort terminally misfolded proteins to the protein degradation machinery (ubiquitin-proteasome system or autophagy) (86).

CHAPERONE PARADIGMS

Research has defined several mechanistic paradigms of chaperone function in protein folding. In the following sections we discuss TF as an ATP-independent chaperone as well as the Hsp70 system, the chaperonins, and Hsp90 as ATP-dependent paradigms. Structural and functional data strongly support the mechanistic models of each of these systems.

Trigger Factor

Bacterial TF is an abundant ~50 kDa protein that binds to ribosomes and interacts with most nascent polypeptides (7, 55, 56, 59, 63, 87, 88). The crystal structure of *E. coli* TF reveals an elongated structure consisting of three domains, an N-terminal ribosome-binding domain, a peptidylprolyl *cis/trans* isomerase (PPIase) domain, and a C-terminal domain (positioned between the N-terminal and PPIase domains) (**Figure 4*a***) (61). The N-terminal

PPIase: peptidylprolyl *cis/trans* isomerase

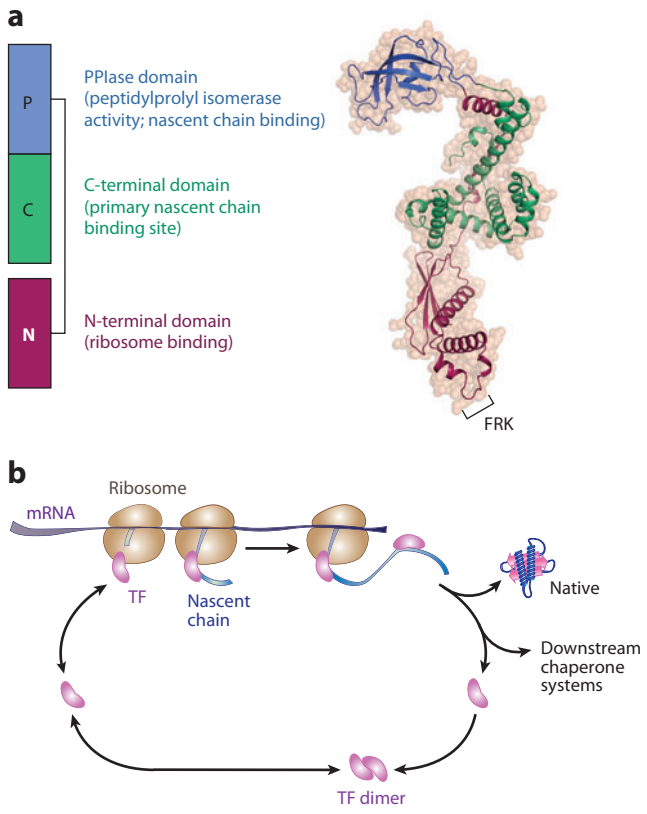


Figure 4

Structure and functional cycle of trigger factor (TF). (*a*) Structure of TF. The N-terminal domain of TF (*magenta*) contains the Phe-Arg-Lys (FRK) ribosome-binding loop and connects by a long linker to the peptidylprolyl *cis/trans* isomerase (PPIase) domain (*blue*). The C-terminal domain (*green*) is in the center of the protein and provides the main binding site for the nascent chain substrate [Protein Data Bank (PDB) 1W26]. (*b*) TF reaction cycle. Free TF exists in the cytosol as a dimer in rapid equilibrium with monomers. Monomeric TF binds to ribosomes with translating nascent chains. Hydrophobic sequence motifs in the nascent chain regulate TF-nascent chain interaction. Binding to the elongating nascent chain may persist as TF dissociates from the ribosome (with $t_{1/2} \sim 10$ s) (63), allowing a second TF molecule to bind to the ribosome at the polypeptide exit site. Concurrent with release from TF, the newly synthesized polypeptide folds into its native state or is transferred to downstream chaperones.

NBD: nucleotide-binding domain

SBD: substrate-binding domain

domain mediates binding of monomeric TF to the large ribosomal subunit at proteins L23 and L29 in close proximity to the polypeptide exit site (60, 61, 89, 90). L23 is essential for TF binding and is thought to signal the progression of the nascent chain through the ribosomal exit tunnel (89), whereas L29 has an auxiliary function (60). The PPIase domain connects to the N domain via a long linker and is most distal to the ribosome docking site. It belongs to the family of FK506 binding proteins and recognizes stretches of eight amino acids that are enriched in basic and aromatic residues (7). Studies have measured PPIase activity in vitro, but its contribution to folding in vivo has remained unclear, as the domain is dispensable for TF function (59, 67, 91, 92). Cross-linking experiments showed that the PPIase domain interacts with longer nascent chains, presumably representing an auxiliary substrate recognition site (62, 87). The centrally located C-terminal domain is structurally similar to the N-terminal domain of the periplasmic chaperone SurA and provides the primary binding site for the nascent chain (87, 93).

Cells contain an excess of TF compared with ribosomes; the non-ribosome-bound fraction is in rapid monomer-dimer equilibrium (**Figure 4*b***) (63, 94). TF monomer aids de novo folding through ATP-independent cycles of binding and release from both the ribosome and the nascent chain. Binding to the ribosome (mean residence time of ~10–15 s) is a prerequisite for interaction with the nascent chain. The disposition of the bound peptide to bury hydrophobic regions during folding likely drives the eventual release of TF from the nascent chain. Accordingly, TF slows hydrophobic chain collapse and delays cotranslational folding (37, 95, 96). Furthermore TF may remain bound to certain polypeptides after their release from the ribosome, which is consistent with a role for TF as a holdase in ribosome assembly (97, 98). Several studies indicate that TF retains a high degree of conformational flexibility on the ribosome during interactions with the emerging nascent chain (62, 63, 89, 90). This flexibility likely enables TF to accommodate a wide range of polypeptides (63, 88). Although it primarily binds hydrophobic chain segments (63, 88), TF also interacts with small basic proteins, including many ribosomal proteins (56, 88, 98).

The Hsp70 System

Hsp70 chaperones are a ubiquitous class of proteins. They are involved in a wide range of protein quality control functions, including de novo protein folding, refolding of stress-denatured proteins, protein transport, membrane translocation, and protein degradation.

Structure and reaction cycle. Hsp70 consists of an N-terminal nucleotide-binding domain (NBD) and a C-terminal substrate-binding domain (SBD) connected by a highly conserved hydrophobic linker region (**Figure 5*a***). The N-terminal domain has an actin-like fold; it consists of two lobes, each containing two subdomains, with the nucleotide-binding cleft situated in between (5, 99). The SBD consists of a β-sandwich subdomain and an α-helical lid with the substrate binding site located in the β-sandwich subdomain (5). The SBD binds to 5–7-residue peptide segments enriched in hydrophobic amino acids and typically flanked by positively charged residues. The peptide binds in an extended conformation mediated by hydrogen bonds between the SBD and the peptide backbone and by van der Waals contacts with the hydrophobic side chains (5, 100).

Conformational changes in the NBD upon ATP binding and hydrolysis are allosterically coupled to the SBD, regulating peptide binding and release. Binding of ATP to the NBD triggers the attachment of the hydrophobic interdomain linker and the α-helical lid of the SBD to the NBD, which opens the peptide binding pocket (101, 111), as initially revealed in structures of the Hsp70 homolog Hsp110 (**Figure 5*a***) (103–105). Hydrolysis of ATP to ADP triggers the detachment of the lid from the NBD and the closing of the SBD; NBD and SBD are loosely held together by the linker in a dynamic random coil conformation (106, 107).

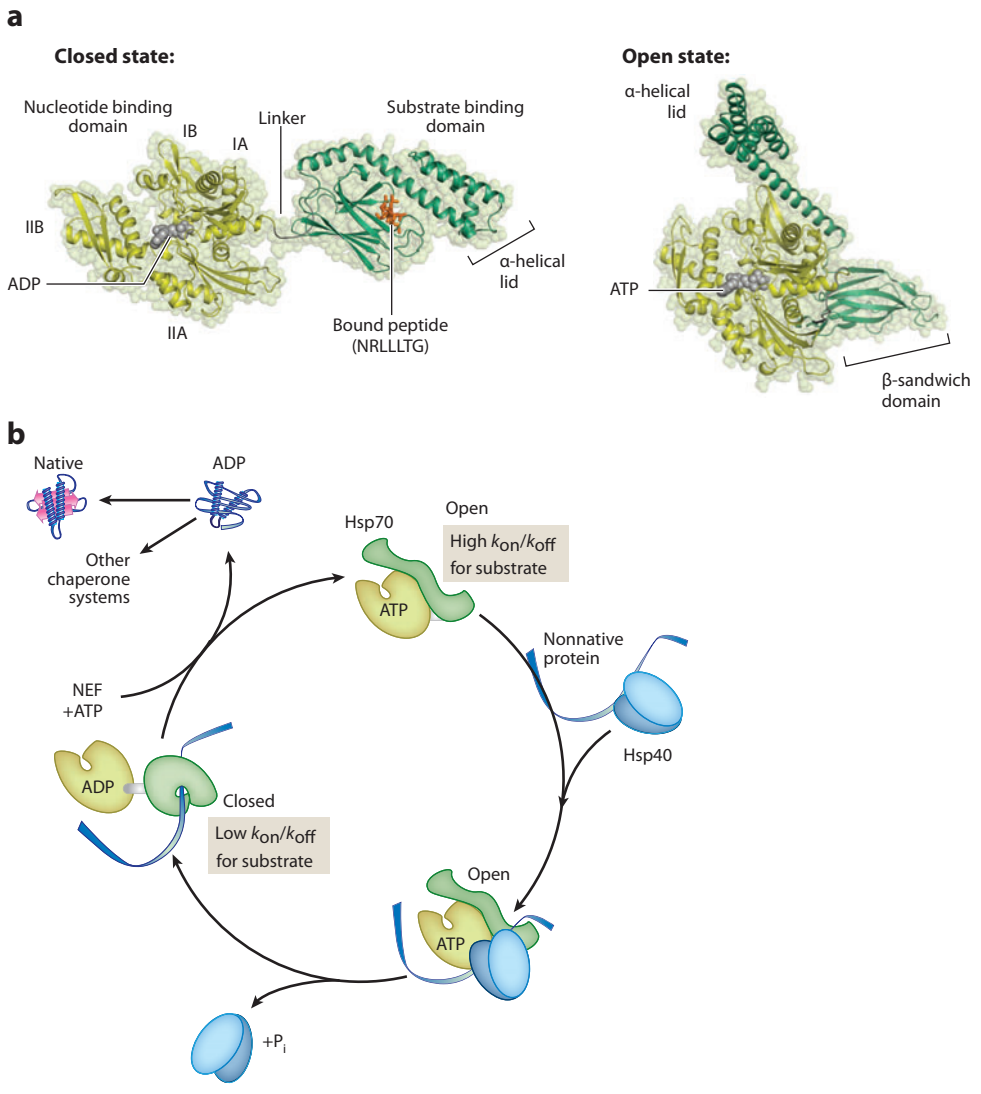


Figure 5

Structure and functional cycle of Hsp70. (*a*) Structure of Hsp70. Hsp70 consists of two domains, the nucleotide-binding domain (NBD) and the substrate-binding domain (SBD), connected by a conserved linker. The closed state of *Escherichia coli* DnaK (*left*; PDB 2KHO) was solved using a combination of solution nuclear magnetic resonance spectroscopy and crystal structures of the individual domains (106). The structure illustrates the ADP-bound NBD separated by a linker from the SBD. The α-helical lid of the SBD is closed over the substrate peptide (NRLLLTG) bound in the pocket of the β-sandwich domain. The open state is illustrated by the crystal structure of ATP-bound Sse1 (yeast Hsp110; *right*; PDB 2QXL). The β-sandwich domain contacts subdomain IA of the NBD, whereas the α-helical lid contacts subdomains IA and IB. (*b*) Reaction cycle. ATP binding to the NBD stabilizes the open state of Hsp70, facilitating the binding of substrate protein recruited to Hsp70 by Hsp40 cochaperone. The open state has fast on and off rates for substrate peptide. Hsp40 stimulates ATP hydrolysis on Hsp70, resulting in the closing of the SBD α-helical lid over the bound substrate peptide. The closed state has slow on and off rates for substrate peptide. NEFs stimulate the release of ADP from the NBD, and ATP binding causes substrate release.

Details of this allosteric mechanism are under intense investigation (108). The ATP-bound open state has high on and off rates for peptide substrate, whereas the ADP-bound closed state has low on and off rates (**Figure 5*b***) (5, 100). In turn, substrate binding increases the rate of ATP hydrolysis (109–111). Substrate release allows folding (i.e., the burial of hydrophobic residues) to proceed. Proteins unable to fold rapidly upon dissociation from Hsp70 may rebind, transfer to downstream chaperones, or be transferred to the degradation machinery.

Cochaperones. Hsp40 (J protein) and NEF cochaperones regulate the Hsp70 reaction cycle (38, 100). The Hsp40 proteins constitute a large family with more than 40 members in humans (75). All of them contain a J domain, which binds to the N-terminal ATPase domain of Hsp70 and the adjacent linker region (112, 113). Canonical Hsp40s (members of classes I and II) function as chaperones independently and recruit Hsp70 to nonnative substrate proteins. Other Hsp40s (class III) are more diverse and combine the J domain with a variety of functional modules (75, 114, 115). The interaction with Hsp70 strongly stimulates (>1,000-fold) the hydrolysis of Hsp70-bound ATP to ADP, resulting in stable substrate binding by Hsp70 in the closed conformation (38, 100). Subsequent binding of an NEF to the NBD of Hsp70 catalyzes the exchange of ADP to ATP, opening the SBD and thereby triggering substrate release (**Figure 5*b***).

Bacteria have one Hsp70 NEF, GrpE, whereas eukaryotic cells contain several structurally unrelated families of NEFs, including the Bcl-2-associated athanogene (BAG) domain proteins, HspBP1 and Hsp110 proteins (116–119). Crystal structures of Hsp70-NEF complexes suggest that all NEFs, except HspBP1, stabilize the Hsp70 NBD in a conformation with an open nucleotide-binding cleft (104, 105, 120–123). The most abundant and ubiquitous eukaryotic NEFs are the Hsp110 proteins, which are structurally related to Hsp70 (103–105). Hsp110 may function as holdases for nonnative proteins and cooperate with Hsp70 and Hsp40 in protein disaggregation (104, 124, 125).

Proper regulation of the ATPase cycle is crucial for efficient Hsp70 function. Hsp40 and NEF proteins are present at lower levels relative to their partner Hsp70. They provide a means of diversifying Hsp70 function and establish substrate specificities for the Hsp70 machinery. For example, the human genome encodes 41 different J-domain proteins compared with 11 Hsp70s and 13 NEFs (75). Interestingly, inactivating mutations in the NEF Sil1 cause the neurodegenerative disease Marinesco-Sjögren syndrome, which highlights the critical importance of the nucleotide exchange function (126, 127).

The Chaperonin System

Chaperonins are large double-ring complexes of 7–9 ~60 kDa subunits per ring. They are unique among molecular chaperones in that they encapsulate their substrate proteins, one molecule at a time, in a central cavity for folding unimpaired by aggregation (4, 5, 17). Two groups of distantly related chaperonins can be distinguished (6, 76, 77).

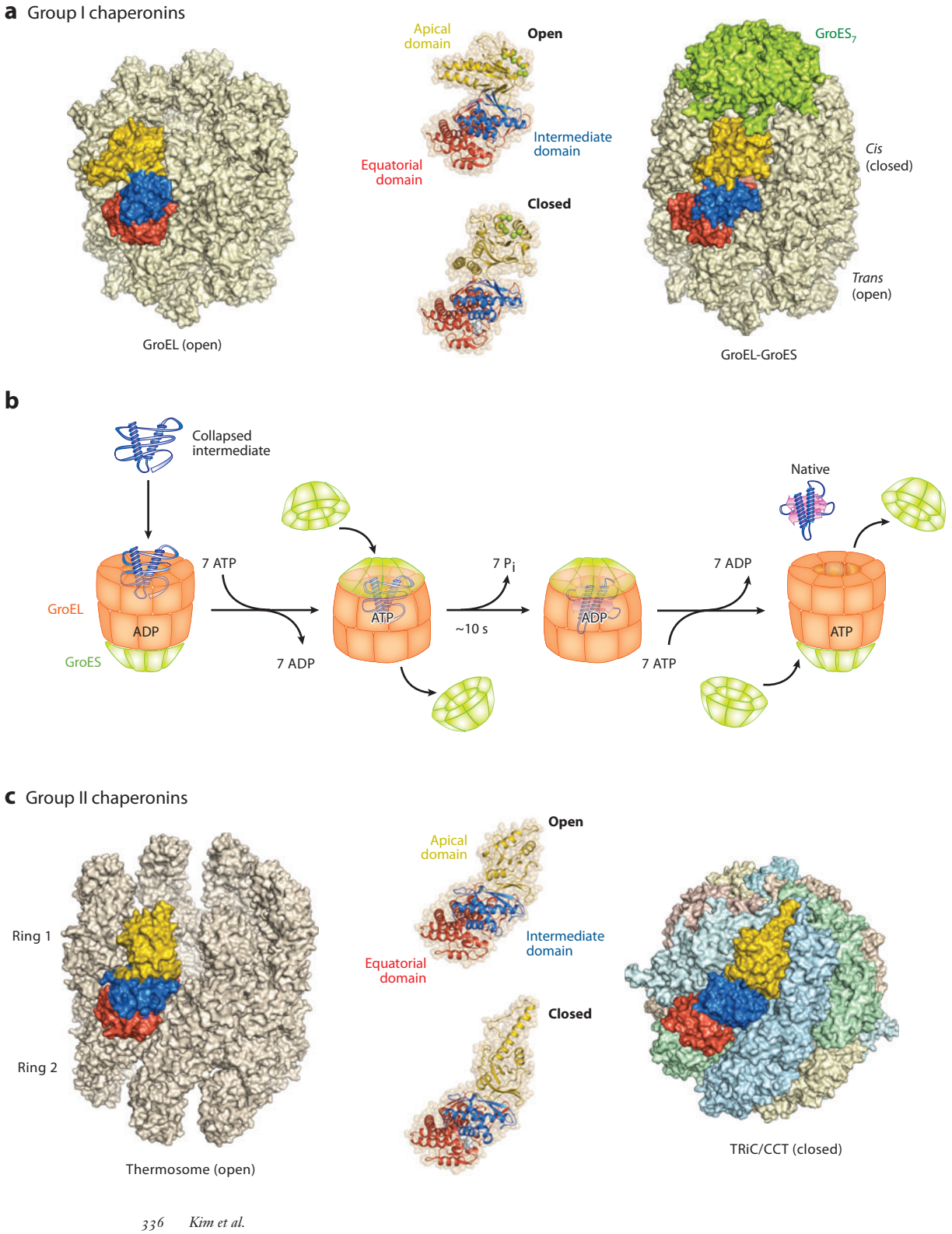
Group I chaperonin GroEL. Group I chaperonins are present in the bacterial cytosol (GroEL), the mitochondrial matrix (Hsp60), and the stroma of chloroplasts (Cpn60). They have seven subunits per ring and are defined by their functional requirement for lid-shaped cochaperones (GroES in bacteria, Hsp10 in mitochondria, and Cpn10/Cpn20 in chloroplasts). The GroEL-GroES system of *E. coli* has been studied most extensively. The subunits of GroEL contain an equatorial ATPase domain, an intermediate hinge domain, and an apical domain (**Figure 6*a***) (77, 100). The apical domains form the entrance to the GroEL cavity and expose hydrophobic amino acid residues, which mediate substrate binding. GroEL-bound substrates typically interact with multiple apical domains (77, 128) and populate an ensemble of compact and locally expanded states that lack stable tertiary interactions, similar to a molten globule (4, 129–131). Binding to GroEL prevents aggregation of these flexible

folding intermediates, and subsequent folding depends critically on the global encapsulation of the substrate in the chaperonin cavity by the cochaperone GroES (7, 132–135). GroES is a heptameric ring of ~10 kDa subunits that binds to the apical GroEL domains, capping the GroEL cylinder (**Figure 6*a*,*b***) (4, 77, 100).

GroEL and GroES undergo a complex binding-and-release cycle that is allosterically regulated by ATP binding and hydrolysis in the GroEL subunits (**Figure 6*b***) (4, 77, 100, 136). Cooperative binding of ATP to GroEL initiates a series of conformational changes that trigger the association of GroES, followed by substrate release from hydrophobic binding sites into a GroES-capped, hydrophilic folding chamber (137). Proteins up to ~60 kDa can be encapsulated and are free to fold in the cage for ~10 s (at 25°C) (132), the time needed for ATP hydrolysis in the GroES-capped ring (*cis* ring). The protein substrate leaves GroEL upon GroES dissociation, which is induced by ATP binding in the opposite ring (*trans* ring) (**Figure 6*b***) (77). Folding intermediates still exposing hydrophobic regions rapidly rebind to GroEL for repeated folding cycles. The exact coordination of the two GroEL rings in the folding cycle is still under investigation (138). Proteins that exceed the size limit of the GroEL-GroES cage may utilize the Hsp70 system for folding (15, 37, 56) or undergo cycles of binding and release from GroEL without GroES encapsulation (77).

In addition to providing an isolated folding environment, other mechanistic elements of the chaperonin cycle contribute to optimizing the rate and yield of the folding process. Repeated events of substrate unfolding in successive binding and release cycles may reverse kinetically trapped states (iterative annealing) (139, 140). Studies have documented unfolding upon binding with Förster resonance energy transfer (FRET) using fluorescence-labeled substrate proteins (130, 140). Additionally, some active unfolding may occur as a result of ATP-dependent movement of the GroEL apical domains (130, 140). However, the significance of iterative annealing is unclear considering that a single round of substrate binding and encapsulation (using a single-ring mutant of GroEL that binds GroES stably) results in substrate protein folding with equal efficiency and kinetics as achieved through multiple cycles of binding and release (7, 132, 141). Whereas partial unfolding upon initial binding may dissociate nonnative interactions in kinetically trapped folding intermediates, the release of protein from the GroEL apical domains may follow a stepwise mechanism in which less tightly bound hydrophobic regions are released first (130). This stepwise release may contribute to avoiding nonnative interactions during protein collapse.

In addition (or as an alternative) to the mechanisms discussed above, growing experimental and theoretical evidence suggests that the GroEL-GroES cage promotes folding by sterically confining folding intermediates (7, 132, 141–145). This model assigns an active role to the chaperonin cage, as opposed to the view that it functions solely as a passive aggregation-prevention device (146). In support of the confinement model, electron microscopy demonstrated that substrates fully occupy the limited volume of the GroEL-GroES cage (134). The resulting constraints on the substrate protein will inevitably affect its folding energy landscape. Indeed, evidence shows that encapsulation in the GroEL-GroES cage accelerates folding up to tenfold compared with the rate of spontaneous folding (measured without interference of aggregation) (7, 132, 141, 147). In addition to steric confinement, mutational analysis demonstrated that the charged residues of the GroEL cavity wall are critical for the observed acceleration (132, 133, 148). According to molecular dynamics simulations, these polar residues accumulate ordered water molecules in their vicinity, thereby generating a local environment in which a substrate protein may bury exposed hydrophobic residues more effectively (144). This would result in the entropic destabilization of flexible folding intermediates in a manner similar to the role of intramolecular disulfide bonds in promoting the folding of secretory proteins. In this context, it is interesting



to note that oxidizing folding compartments supporting disulfide bond formation (e.g., the bacterial periplasm or ER) do not contain chaperonins (141).

Obligate GroEL substrates typically have complex α/β or α + β domain topologies, such as the $(\beta/\alpha)_8$ TIM barrel fold (15, 46, 53), which are stabilized by many long-range interactions. Such proteins must navigate complex folding energy landscapes and overcome entropic barriers to reach their native states (141); hence, they may benefit from steric confinement during folding. Moreover, obligate GroEL substrates often have relatively low sequence hydrophobicity, consistent with inefficient hydrophobic collapse and population of aggregation-prone intermediates that are recognized by GroEL (46, 141, 149). Recent evidence indicates that GroEL-GroES can also accelerate the folding of proteins containing trefoil-knotted structures, a complex topological arrangement that would plausibly form more efficiently in a confining environment (150). Future studies are needed to define precisely what structural properties distinguish GroEL-dependent from GroEL-independent proteins.

Group II chaperonin TRiC/CCT. Although all chaperonins share a common cylindrical architecture, substantial structural differences exist between group I and group II chaperonins (**Figure 6*a*,*c***). For example, group II chaperonins have apical protrusions that function as a built-in lid in place of a separate GroES-like cochaperone. Also, group II chaperonins contain eight or nine subunits per ring, which are stacked directly opposite one another in the two rings (77, 151–158). In contrast, each of the seven subunits of group I chaperonins interdigitates between two subunits of the opposite ring. In many cases, group II chaperonins are hetero-oligomeric, containing up to eight paralogous subunits per ring in a defined order in the case of the eukaryotic chaperonin TRiC (159, 160). Similar to GroEL, group II chaperonins also cycle between open and closed states, and substrate encapsulation is essential for folding (161). But in contrast to GroEL, ATP hydrolysis, not ATP binding, triggers cavity closure, and ATP hydrolysis transition state analogs (e.g., ADP-aluminum fluoride) stabilize the closed state (136, 161, 162). During the transition to the closed state, the apical domains of adjacent subunits undergo pairwise

Figure 6

Structure and function of the chaperonin systems. (*a*) Structure of group I chaperonin. The crystal structures of GroEL (PDB 1SS8) (*left*) and the asymmetrical GroEL (7 ADP)-GroES complex (PDB 1PF9) (*right*) appear with GroES shown in green and one subunit of GroEL colored to indicate its domain structure (equatorial nucleotide-binding domain in *red*; intermediate hinge domain in *blue*; and the apical substrate and GroES-binding domain in *yellow*). The conformational differences between the GroEL subunits in the open state (GroEL and *trans* ring of GroEL-GroES complex) and in the closed state (*cis* ring of GroEL-GroES complex) appear in ribbon representations of single subunits (*middle*). The green spheres represent hydrophobic residues on helices 8 and 9 of the apical domain that are involved in substrate binding in the open conformation and in GroES binding in the closed state. (*b*) GroEL-GroES reaction cycle. Substrate protein as a collapsed folding intermediate is bound by the open GroEL ring of the asymmetrical GroEL-GroES complex, shown in panel *a*. Binding of ATP to each of the seven GroEL subunits causes a conformational change in the apical domains, which results in the exposure of the GroES binding residues, allowing substrate encapsulation in the *cis* complex. ADP and GroES dissociate from the opposite ring (*trans* ring) together with the previously bound substrate. The newly encapsulated substrate is free to fold in the GroEL cavity during the time needed to hydrolyze the seven ATP molecules bound to the *cis* ring (~10 s). ATP binding followed by GroES binding to the *trans* ring triggers GroES dissociation from the *cis* ring, releasing the substrate protein. (*c*) Structure of group II chaperonin. The crystal structures of the open form of the homo-oligomeric *Methanococcus maripaludis* thermosome (PBD 3KFK) (*left*) and the closed form of TRiC/CCT (PDB 4D8Q) (*right*) are shown. The eight paralogous TRiC/CCT subunits appear in different colors, showing both homotypic (*blue* subunits) and heterotypic contacts (*green-beige*, *blue-beige*) between the top and bottom rings. One subunit (CCT3 in the case of TRiC) is colored to indicate its domain structure (equatorial nucleotide-binding domain in *red*; intermediate hinge domain in *green*; and the apical substrate-binding domain in *yellow*). The conformational differences between the open and the closed state, taken from the thermosome crystal structures (PBD 3KFK for the open and PDB 3KFB for the closed state) (*middle*), appear in ribbon representation. In place of a GroES-like cochaperone, the group II chaperonins have an extended apical domain that functions as a built-in lid.

association, resulting in intermediate structures with pseudo-fourfold symmetry (158). Closure is completed when the tips of the helical protrusions form a mixed eight-stranded β-barrel structure around the apical pore.

TRiC interacts with a wide range of cytosolic proteins (49, 50). Prominent substrates include actin and tubulin, which are strictly dependent on the chaperonin for folding (4, 77). A recent crystal structure of bovine TRiC in the open conformation bound to a folding intermediate of tubulin suggests that the substrate initially interacts with loops in the apical and equatorial domains of the TRiC subunits exposed toward the central cavity (157). Helical motifs at the interface between adjacent apical domains have also been implicated in substrate interactions (163). Differences in binding specificities among the different subunits may be important in binding and folding a range of structurally diverse proteins. Accordingly, all eight TRiC subunits are essential in *S. cerevisiae*. Interestingly, the cavity wall exhibits a pronounced segregation of positive and negative surface charges into opposing halves, a feature that might be functionally important (160). The reaction cycle of TRiC is slower than that of GroEL, providing a substantially longer period of protein encapsulation and folding in the cage (164). Also, the iris-like closing mechanism enables the encapsulation of TRiC-dependent domains in the context of large multidomain proteins that cannot be encapsulated in their entirety (164a). Such a mechanism would circumvent the size constraints of chaperonin-assisted folding and may have facilitated the evolution of eukaryotic multidomain proteins with complex architectures. TRiC also interacts with N-terminal fragments of mutant huntingtin that contain an expanded polyglutamine repeat sequence (165–168). Binding to TRiC modulates the aggregation properties of this protein and reduces its cytotoxicity.

The Hsp90 System

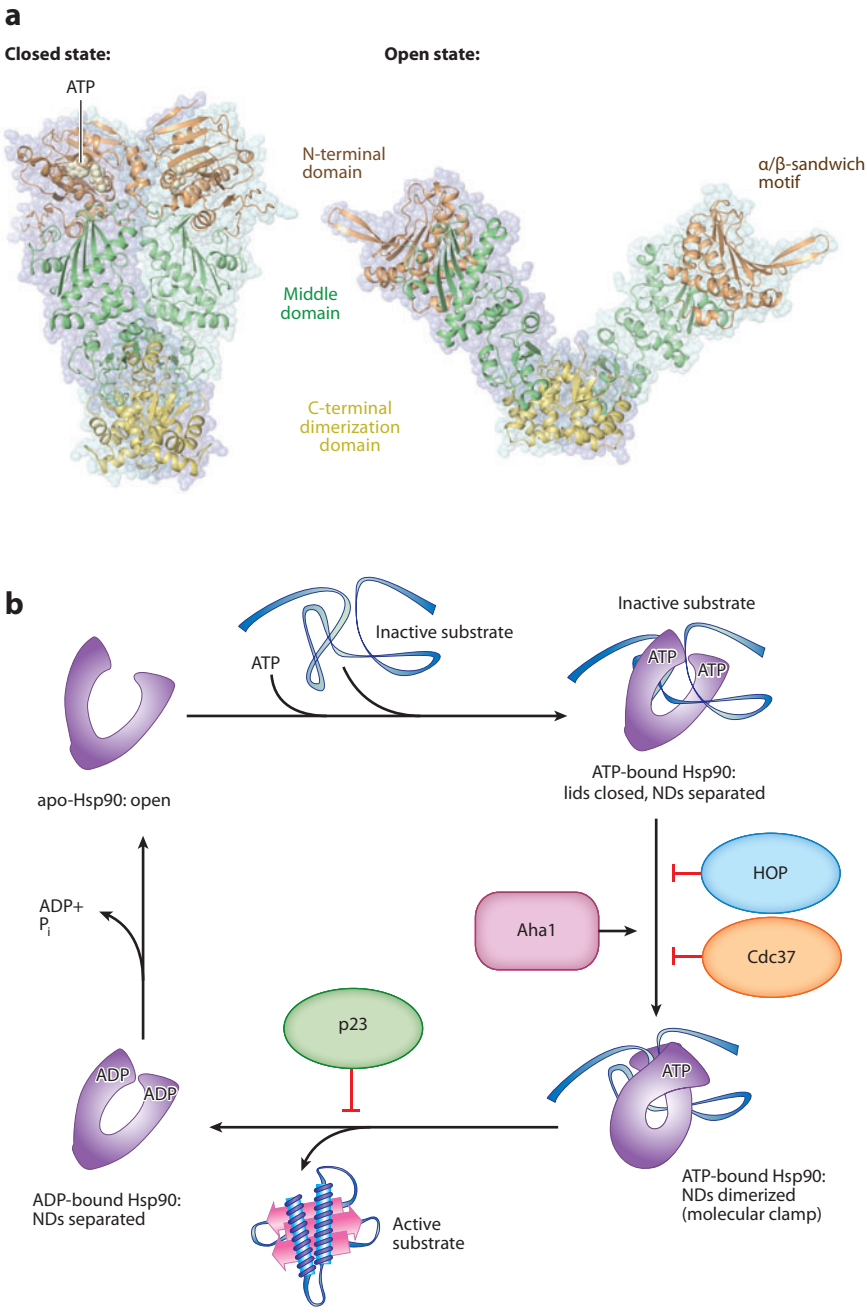
The Hsp90 chaperone system has a central role in cell regulation. Among its substrates are multiple signaling molecules, which are delivered to Hsp90 by Hsp70 chaperones and other cofactors.

Structure and reaction cycle. The Hsp90 chaperones structurally belong to the gyrase, histidine kinase, and MutL superfamily of ATPases (169). Crystal structures of homodimeric Hsp90 molecules from bacteria (170), yeast (171), and mammals (172) illustrate both open and closed functional states of the chaperone (**Figure 7*a***). In addition, complete or partial structures of Hsp90 in complex with different cochaperones or small molecule inhibitors were obtained by X-ray crystallography and cyroelectron microscopy (81, 169, 171, 173–176). Collectively, these structures reveal a high degree of flexibility in Hsp90 conformations (177), consistent with the diversity of Hsp90 client proteins (45).

Figure 7

Structure and functional cycle of the Hsp90 system. (*a*) Structure of Hsp90. Crystal structures of the Hsp90 dimer in the ATP-bound closed state (*Saccharomyces cerevisiae*; PDB 2CG9) (*left*) and the nucleotide-free open state (*E. coli*; PDB 2IOQ) (*right*) are shown with the nucleotide-binding N-terminal domain in orange, middle domain in green, and C-terminal domain in yellow. (*b*) Hsp90 reaction cycle. Inactive substrate protein binds to ATP-bound Hsp90. In this state the ATP lids are closed and the N-terminal domains are separated. In the next step, the N-terminal domains dimerize, forming the closed Hsp90 dimer (referred to as a molecular clamp) with twisted subunits. This metastable state is committed to ATP hydrolysis, upon which the N-terminal domains dissociate. The bound substrate protein is conformationally activated as Hsp90 proceeds through the cycle. Cofactors such as Cdc37 and the Hsp90 organizing protein (HOP) slow the ATP hydrolysis step of the cycle, whereas the activator of Hsp90 ATPase (Aha1) enhances ATP hydrolysis. The cofactor p23 stabilizes the closed dimer to slow the release of substrate protein from Hsp90.

Hsp90 consists of three domains: the highly conserved N-terminal domain, the middle domain, and the C-terminal domain (**Figure 7*a***) (169). The N-terminal domain contains the ATP binding site, which comprises a two-layer α/β-sandwich structure. Inhibition of ATP binding and hydrolysis either by mutagenesis of the N domain or by specific inhibitors, such as geldanamycin and radicicol, demonstrated the functional requirement of the Hsp90



Proteostasis network (PN): the collection of cellular components involved in proteostasis maintenance

ATPase (81, 169). The N-terminal domain contains a long, highly charged loop segment with regulatory function located in proximity to the middle domain (178, 179). The middle domain, composed of α-β-α motifs, is essential for interactions with substrate proteins and regulation of ATP hydrolysis (179a, 180, 181). The C-terminal domain of Hsp90 contains the interaction site for the assembly of the functional Hsp90 dimer, a mixed four-helix bundle (173). The extreme C terminus of Hsp90 contains the MEEVD sequence motif, which mediates interactions with numerous cochaperones containing TPR domains (81).

Despite an abundance of structural information, the detailed mechanism by which Hsp90 recognizes kinases and other substrate proteins and facilitates their folding or conformational regulation is not well understood. Similar to other chaperones, the Hsp90 dimer undergoes an ATP-regulated reaction cycle accompanied by extensive structural rearrangements (**Figure 7*b***) (100, 182). In this process, the open, V-shaped state of Hsp90 receives inactive client protein and then converts into the closed form, often described as a molecular clamp formed by the N domains (176, 182). This reaction is driven by the combined effects of ATP binding, ATP hydrolysis, posttranslational modifications (183, 184), and interactions with multiple cochaperones (**Figure 7*b***). Formation of the closed conformation results in a compaction of the Hsp90 dimer in which the individual subunits twist around each other (171). After hydrolysis the Hsp90 N termini separate, releasing the client protein in an active state (**Figure 7*b***). Recent electron microscopy and FRET studies using ATP and nonhydrolyzable ATP analogs suggest that the N-terminally clamped, closed state and the open conformation are in a more dynamic equilibrium than previously believed (185–187).

Cochaperones. Hsp90 intimately cooperates with multiple cochaperones that regulate different steps of the reaction cycle (**Figure 7*b***). Cochaperones HOP (Sti1) and Cdc37 (p50) stabilize the open conformation of the Hsp90 dimer (81, 174, 175, 188), inhibit ATP hydrolysis, and facilitate substrate protein binding. HOP mediates client transfer from the Hsp70-Hsp40 system, whereas Cdc37 functions as an adapter for kinases. Conversely, p23 (Sba1) couples the Hsp90 ATPase activity to efficient polypeptide dissociation. Two molecules of p23 bind to the N domains of closed Hsp90 and presumably stabilize the ATP-bound state (81, 171, 189). The activator of Hsp90 ATPase (Aha1) binds asymmetrically to the Hsp90 middle domain, stimulating ATP hydrolysis and inducing transition to the closed state (180, 181, 185, 190). In addition, Hsp90 interacts via its C terminus with a range of TPR domain–containing cochaperones. These factors often contain PPIase domains (Cyp40, FKBP51, and FKBP52) and participate in Hsp90-mediated client protein folding (45).

PROTEOSTASIS NETWORK

The successful folding of newly synthesized proteins and their conformational maintenance are essential in sustaining a functional proteome. In addition, the cellular concentration, localization, and activity of each protein must be carefully controlled in response to intrinsic and environmental stimuli. Although research has made major advances in elucidating the mechanics of individual chaperones, we are far from understanding how the various chaperone systems cooperate as a network and function in conjunction with the protein transport and degradation machineries to ensure proteome integrity. The term proteostasis describes this state of healthy proteome balance, whereas proteostasis network (PN) refers to the collection of cellular components involved in proteostasis maintenance (8). Failure of proteostasis is implicated in disease and the deleterious effects of aging (10). Molecular chaperones, through their ability to recognize incorrectly folded proteins, have multiple key roles in the PN (**Figure 8*a***).

The PN is regulated by interconnected pathways that respond to specific forms of

cellular stress, including the cytosolic heat shock response (HSR) (191), the unfolded protein response (UPR) in the endoplasmic reticulum (192), and the mitochondrial UPR (193). Additionally, PN regulation is integrated with pathways involved in inflammation, response to oxidative stress, caloric restriction/starvation, and longevity. The PN of mammalian cells consists of ~1,300 different proteins involved in protein biogenesis (~400), conformational maintenance (~300), and degradation (~700), with many proteins being part of more than one pathway (**Figure 8*b***). Different cell types vary in their proteostasis capacity and thus in their stress sensitivity and vulnerability to protein aggregation (194, 195).

Significance of Conformational Maintenance

After their initial folding and assembly, many proteins remain reliant on molecular chaperones throughout their cellular lifetime to maintain their functionally active conformations. This is consistent with the notion that proteins with key cellular functions are often structurally dynamic and may be expressed at levels at which they are poorly soluble (14, 46). Many of the chaperone systems discussed in the previous sections function not only in de novo folding but also in conformational maintenance, i.e., they prevent aggregation of misfolded proteins and mediate their refolding. Specific proteins may interact with as many as 25 different types of chaperones throughout their lifetime, as shown in yeast (51). Pulse-chase labeling and quantitative proteomics have described the contributions of the bacterial Hsp70 and chaperonin systems to conformational maintenance (15, 56). Upregulation of chaperones under conditions of conformational stress, such as heat shock or oxidative stress, expands the cellular capacity for the prevention of aggregation. Failure of conformational maintenance is particularly relevant to the onset of age-related degenerative disorders, which typically involve protein aggregation (9).

Degradation

A central feature in the organization of the PN is the tight interconnection of molecular chaperone functions with the pathways of protein degradation, which serve to remove nonfunctional, misfolded, or aggregated proteins that may otherwise disrupt proteostasis. The PN branch of degradation includes the ubiquitin-proteasome system (UPS) and machinery of autophagy (23, 196–200). Approximately 700 proteins are implicated in protein degradation, reflecting the fundamental importance of these pathways in cell regulation and protein homeostasis.

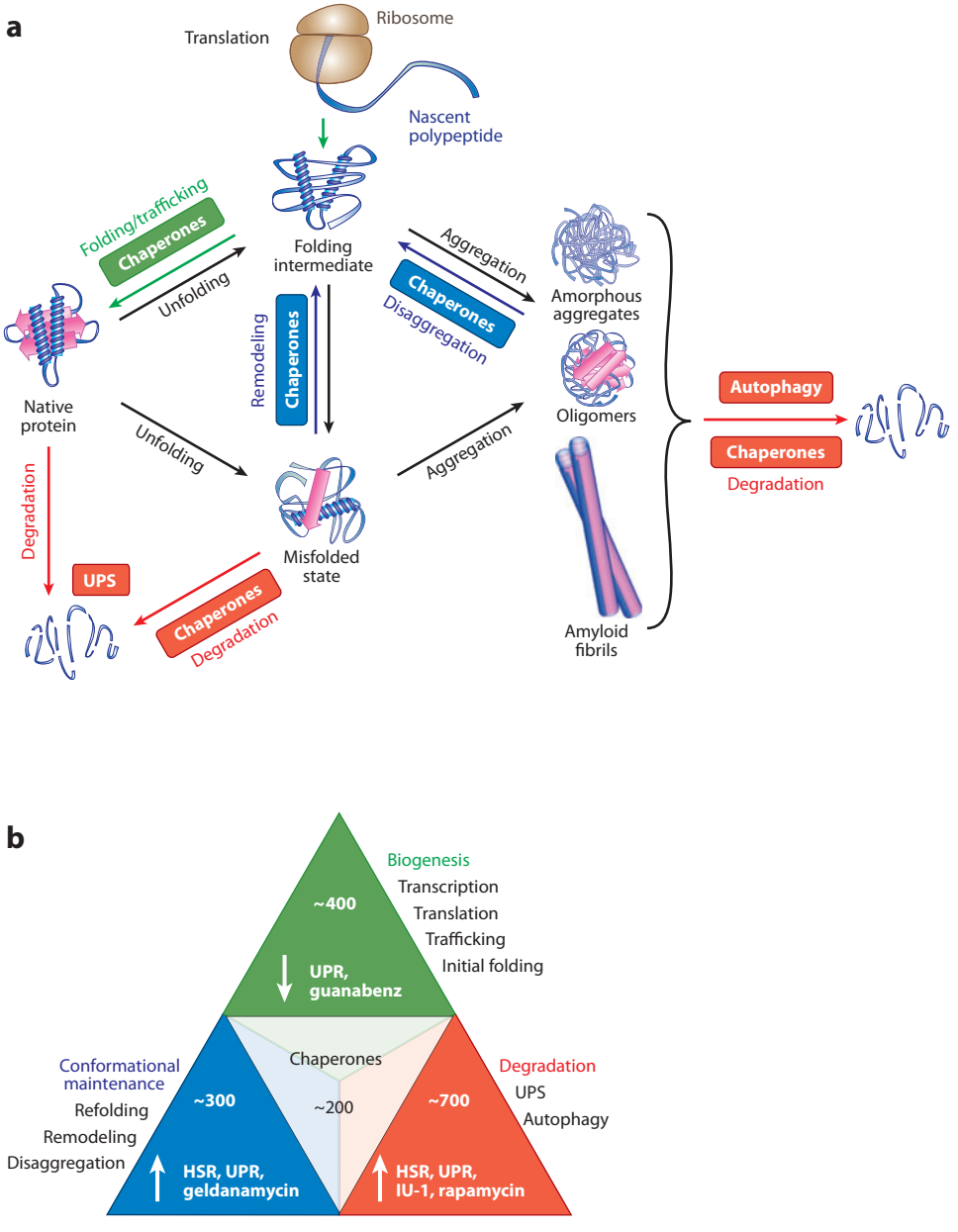
UPR: unfolded protein response

UPS: ubiquitin-proteasome system

Degradation via the UPS depends on protein unfolding by the 26S proteasome (201) and generally requires that chaperones maintain target proteins in a nonaggregated state. Chaperones cooperate with various E3 ubiquitin ligases in recognizing and targeting misfolded proteins for proteasomal degradation. For example, Hsp70 and Hsp90 cooperate with the U-box-dependent ubiquitin ligase CHIP (C terminus of Hsc70-interacting protein) and a variety of other cofactors (such as BAG1 and BAG3) to ubiquitinate client proteins (23, 86, 198). CHIP interacts with either Hsc70 or Hsp90 via its TPR domain (86, 198, 202). CHIP also cooperates with the E2 enzyme Ubc13-Uev1a to form noncanonical Lys63-linked polyubiquitin chains, which suggests an additional role in targeting proteins for destruction via autophagy (202, 203). BAG1 and BAG3 associate (via their BAG domain) with the NBD of Hsp70 and also interact with CHIP to promote the ubiquitination of Hsp70-bound client proteins (86, 198, 204). BAG1 targets proteins for degradation by the UPS, whereas BAG3 mediates degradation by macroautophagy.

Aggregated proteins that cannot be unfolded for proteasomal degradation may be removed by autophagy and lysosomal/vacuolar degradation. Loss of autophagy causes inclusion body formation and neurodegeneration, even in the absence of additional stress, demonstrating the importance of this pathway for proteostasis (205). Aggregate removal by

autophagy entails the sorting and concentration of small protein aggregates to specific sites in the cytosol (206–209) with the participation of chaperones such as Hsp42 (small Hsp) (209). These deposition sites include the aggresome, to which components mediating autophagic vacuole formation are recruited (206, 210, 211). Additional pathways of autophagy include chaperone-mediated autophagy (CMA) and chaperone-assisted selective autophagy (CASA) (198, 200, 212). In CMA, Hsc70 and certain cochaperones bind to a KFERQ sequence motif present in approximately 30% of all cytoplasmic proteins. This complex binds the lysosomal membrane protein LAMP2, followed by translocation of the substrate



protein across the lysosomal membrane for degradation (200). Whereas CMA is ubiquitin-independent, CASA uses ubiquitination as a signal for degradation in a pathway that involves the ubiquitin ligase CHIP, Hsc70, and BAG3, as well as the autophagic ubiquitin adaptor p62 (213). CASA is reportedly required for the removal of damaged skeletal muscle proteins such as filamin (213). Notably, chaperones such as Hsp70/Hsc70 act in parallel in the different branches of the PN, and our understanding of how they switch their function between initial folding and conformational maintenance to degradation is still rudimentary.

Proteostasis Collapse in Aging and Disease

As shown in *Caenorhabditis elegans*, *Drosophila*, and the mouse, the ability of cells and tissues to maintain proteostasis declines during aging, concurrent with the capacity to respond to conformational stresses (214–220). Why this is the case is still unclear, but one proposed explanation is that multicellular organisms place less value on protecting the somatic proteome against internal and external stress once propagation of the germ line is certain. The gradual decline in proteostasis capacity would then result in the accumulation of misfolded (or oxidized) proteins, leading to the deposition of aggregates, cellular toxicity, and cell death (214, 219, 221). Accordingly, age is a universal risk factor for a range of degenerative diseases associated with protein misfolding and aggregate deposition.

The diseases of aberrant protein folding associated with aging are usually categorized as toxic gain-of-function disorders and include type 2 diabetes and the major neurodegenerative diseases (Parkinson's, Huntington's, and Alzheimer's disease, as well as amyotrophic lateral sclerosis) (17, 222). They either occur sporadically or are dominantly inherited. Classical examples of the latter type are Huntington's disease and related disorders, in which the age of onset is inversely correlated with the length of an expanded polyglutamine tract in the disease protein (214). As aggregation propensity increases with polyglutamine length, manifestation of neuronal degeneration may occur when available proteostasis capacity is no longer sufficient to prevent the formation of toxic aggregates. This is supported by experiments demonstrating that the onset of polyglutamine toxicity in *C. elegans* correlates with age-dependent proteostasis decline (214, 215, 217, 220). The accumulation of protein aggregates in turn exerts pressure on the PN, further accelerating its decline. This is exemplified by the observation that toxic polyglutamine repeat proteins interfere with normal protein clearance by the UPS and the conformational maintenance of metastable proteins by the chaperone network (17, 195, 223, 224). Furthermore, toxic protein aggregation also

Figure 8

The proteostasis network. (*a*) Protein fates in the proteostasis network (PN). The PN integrates chaperone pathways for the folding of newly synthesized proteins, the remodeling of misfolded states, and disaggregation with protein degradation. Panel *a* adapted and modified from Reference 16. (*b*) Central role of molecular chaperones. The three branches of the proteostasis network are interconnected by the functions of molecular chaperones. The approximate number of proteins in each branch as well as the number of chaperone components (including cofactors) is indicated. Activation of the cytosolic heat shock response (HSR) and the unfolded protein response (UPR) of the endoplasmic reticulum generally increases proteostasis capacity in all three branches. Various heat shock factor 1 activators can pharmacologically induce the HSR (38, 229, 230). Treatment with guanabenz results in attenuation of translation and increases proteostasis capacity by reducing the load of potentially misfolding proteins (226). The small molecule compound IU-1 inhibits protein deubiquitination and increases degradation by the ubiquitin-proteasome system (UPS) (236). The drug rapamycin activates autophagy by inhibiting the kinase mammalian target of rapamycin (mTOR) (199).

compromises the cellular response to stress stimuli (221).

The Proteostasis Network as a Drug Target

Considering the impact of proteostasis imbalance in age-related degenerative diseases, returning the cell to a more youthful state by pharmacologically boosting proteostasis capacity is a promising therapeutic strategy. Whereas ligand compounds can stabilize specific disease proteins against aggregation (225), activating proteostasis could benefit a wide variety of diseases and might also delay the deleterious effects of aging (8). In principle, we might achieve this by manipulating the three branches of the PN: biogenesis, conformational maintenance, and degradation—either individually or in combination (**Figure 8*b***). In the biogenesis branch, attenuation of translation may be beneficial by reducing the load of misfolding proteins. The antihypertensive drug guanabenz demonstrates this; besides being an α_2 receptor antagonist, it also stabilizes the translation initiation factor 2 (eIF2) in its inactive, phosphorylated state (226). Furthermore, boosting cellular chaperone capacity can increase the efficiency of folding or degradation of proteins carrying destabilizing mutations and inhibit their aggregation (227). For example, small molecules (e.g., geldanamycin) that activate heat shock factor 1, the main transcriptional regulator of the cytosolic stress response, increase the effective concentration of cytosolic chaperones and suppress the aggregation of various disease proteins (8, 38, 228–230). This approach is based on multiple lines of evidence demonstrating that overexpression of chaperones such as Hsp70 and Hsp40 prevents the aggregation and toxicity of huntingtin and α-synuclein (38, 231–234). The Hsp70 system acts synergistically with the cytosolic chaperonin TRiC to prevent aggregation of proteins with expanded polyglutamine tracts (165–168). Finally, activating the UPS or inducing autophagy can increase the clearance of potentially toxic proteins (**Figure 8*b***) (212, 235, 236).

OUTLOOK

Studies over the past decade have revealed fascinating insights into the structures of a variety of chaperone systems and the mechanisms by which they assist in protein folding. However, most of these advances are derived from analyses in vitro, and consequently, our understanding of how the pathways of folding in the cell differ from those studied in the test tube is still incomplete. Moreover, for most newly synthesized proteins, the relevant quantitative parameters of folding (rate, yield, and overall efficiency) are unknown. Likewise, we are just beginning to understand how the cellular environment influences protein folding and stability and how translation affects the folding process. For example, what is the role of translational pausing in protein folding and trafficking? Much future work will also be directed toward developing an integrated view of the different aspects of the PN, with particular regard to the cooperation between folding and degradation machineries. Solving this problem will require a broad systems biology approach relying on a combination of ribosome profiling, quantitative proteomics, and computational modeling. How do cells react to conformational stresses or proteostasis deficiencies at the proteome level? Which proteins are prone to misfolding, and why do certain proteins aggregate into toxic species whereas others get degraded? How does the composition of the proteome change during aging, what are the signatures of a youthful proteome, and can we find ways to preserve it longer as we age? Addressing these and related questions offers not only a deeper understanding of cell biology but also the prospect of great medical benefits should we be able to intervene in the numerous, presently incurable diseases of protein aggregation and proteostasis deficiency.

SUMMARY POINTS

1. The efficient biogenesis of proteins in the densely crowded cellular environment depends on molecular chaperones to avoid protein misfolding and aggregation.
2. Chaperones promote folding and inhibit aggregation through cycles of binding and release of nonnative proteins (often ATP regulated) that allow kinetic partitioning.
3. Different classes of molecular chaperones may cooperate in sequential pathways.
4. Nascent-chain-binding chaperones prevent misfolding during translation; folding occurs either immediately upon completion of synthesis or after transfer to downstream chaperones, such as the chaperonins, which complete the folding process.
5. Major ATP-dependent chaperone paradigms in the cytosol include the Hsp70 and Hsp90 systems as well as the chaperonins.
6. The chaperonins are cylindrical, ATP-dependent folding machines that encapsulate a single protein chain, allowing it to overcome kinetic folding barriers while being protected against aggregation.
7. Molecular chaperones function as central elements of the large cellular network of proteostasis control, which comprises the protein biogenesis machinery as well as the ubiquitin proteasome and autophagy systems for protein degradation.
8. Understanding the organization of this network and its regulation during stress and aging will help in developing new strategies for the treatment of a range of age-related degenerative diseases associated with protein aggregation.

FUTURE ISSUES

1. How do the pathways of protein folding in the cell differ from those studied in vitro and how does translation affect the folding process?
2. Can we determine the rates, yields, and overall efficiencies of protein folding at the proteome level?
3. How do molecular chaperones of the various branches of the PN cooperate in maintaining proteome integrity?
4. How does the proteome composition change during proteostasis, stress, and aging?
5. What are the mechanisms underlying the cellular toxicity of protein misfolding and aggregation?
6. Can pharmacological chaperone activation serve as a strategy to combat diseases associated with protein misfolding and aggregation?

DISCLOSURE STATEMENT

F.U.H. and M.S.H. are paid consultants of Proteostasis Therapeutics, Inc.

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