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Recent advances in understanding catalysis of protein folding by molecular chaperones

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Molecular chaperones are highly conserved proteins that promote proper folding of other proteins *in vivo*. Diverse chaperone systems assist *de novo* protein folding and trafficking, the assembly of oligomeric complexes, and recovery from stress-induced unfolding. A fundamental function of molecular chaperones is to inhibit unproductive protein interactions by recognizing and protecting hydrophobic surfaces that are exposed during folding or following proteotoxic stress. Beyond this basic principle, it is now clear that chaperones can also actively and specifically accelerate folding reactions in an ATP-dependent manner. We focus on the bacterial Hsp70 and chaperonin systems as paradigms, and review recent work that has advanced our understanding of how these chaperones act as catalysts of protein folding.

Keywords: chaperonin; confinement; DnaK; GroEL; Hsp40; Hsp60; Hsp70; molecular chaperones; protein folding; protein misfolding

Pioneering experiments by Anfinsen in the 1950s [1] demonstrated that a small protein can fold spontaneously in the absence of additional factors *in vitro*. Subsequent work over the last 50 years has provided detailed insight into the general principles that govern protein folding. The conformational search for the native state is thought to follow a funnel-shaped energy landscape, driven by the burial of hydrophobic residues, and the relative stability of native-like interactions that nucleate the folding reaction [2,3] (Fig. 1). However, a unifying mechanism for protein folding remains elusive [4]. Current models are not generally predictive for protein folding pathways, even if substantial progress has been made toward prediction of protein folds [5].

Several factors complicate the folding process. The folding free-energy landscape is rugged: Protein chains must traverse substantial energy barriers en route to the native state and consequently populate folding intermediates (Fig. 1). Off-pathway intermediates and kinetic traps slow folding, and non-native intramolecular interactions can lead to stably misfolded states [6]. Moreover, folding intermediates expose hydrophobic surfaces that can engage in nonfunctional intermolecular interactions enabling aggregation (Fig. 1). Biophysical studies of protein folding typically focus on small model proteins (often < 100 amino acids) that are simple to express recombinantly and show robust reversible folding *in vitro* [7,8]. The intrinsic challenges associated with folding of the larger, structurally more

Abbreviations
FLuc, firefly luciferase; H/DX-MS, hydrogen/deuterium exchange mass spectrometry; Hsp, heat shock protein; NBD, nucleotide-binding domain; NEF, nucleotide exchange factor; NMR, nuclear magnetic resonance; SBD, substrate-binding domain; TIM, triose-phosphate isomerase.

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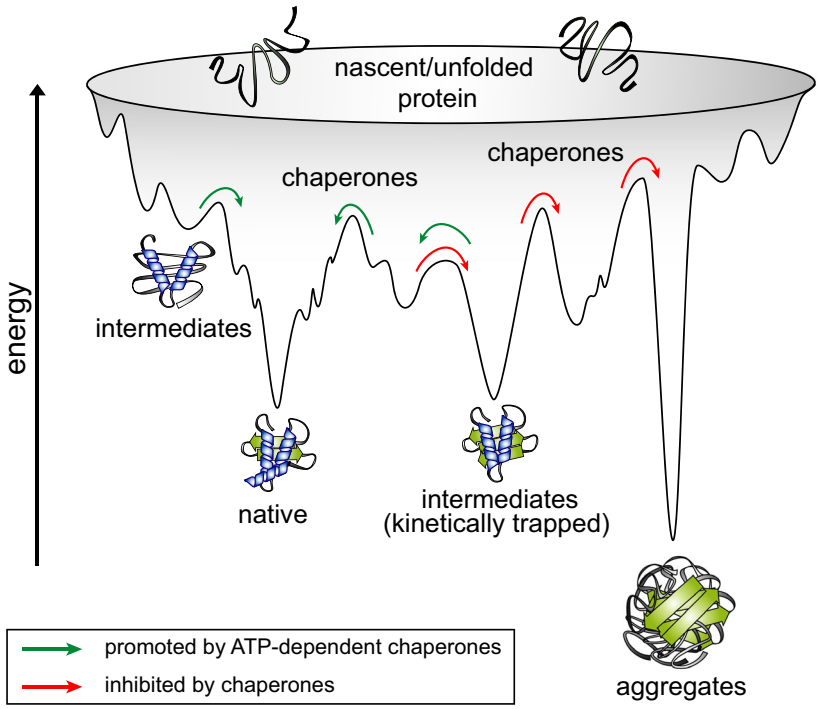

Fig. 1. Molecular chaperones shape the energy landscape of protein folding. During folding, proteins navigate a rugged, funnel-shaped potential free-energy surface en route to the native state. The accumulation of on- and off-pathway intermediates slows folding and entails the risk of misfolding into kinetically trapped states that are prone to form thermodynamically stable aggregates. Molecular chaperones inhibit aggregation, resolve kinetically trapped conformations, and provide kinetic assistance to folding by lowering free-energy barriers that separate folding intermediates from the native state. Figure modified from Ref. [9].

complex proteins that constitute the majority of proteomes [9] may be more pronounced than current folding models suggest, with misfolding being the rule rather than the exception.

The folding problem is exacerbated by conditions *in vivo*. The high concentration of macromolecules in the cell enhances the tendency of non-native proteins to aggregate [10], while proteotoxic stress destabilizes the native state. Moreover, protein folding occurs in the context of translation [11], which entails that nascent polypeptides are exposed to the cellular environment in an incomplete state lacking structural information needed for stable folding [12,13]. Molecular chaperones have evolved in response to these challenges and have in turn contributed to the diversity of proteomes in both prokaryotes and eukaryotes [14,15]. Chaperone classes use variations of a common mechanism of action based on transient binding of sequences enriched in hydrophobic residues. This activity serves to inhibit aggregation, but can also influence the intramolecular interactions that define a protein's folding pathway. Recent research has advanced the idea that molecular chaperones can modulate folding energy landscapes. Here, we discuss examples of folding catalysis by the ATP-dependent Hsp70 and chaperonin (Hsp60) class of chaperones, and explore possible underlying mechanisms.

Catalysis of folding by the Hsp70 chaperone system

Chaperones of the Hsp70 class (DnaK in bacteria) are highly allosteric molecular machines that participate in a range of cellular processes, including protein folding and refolding, trafficking, translocation, disaggregation, and degradation [16–18]. These diverse activities exploit the affinity of Hsp70 for short (5–7 amino acid) sequence elements enriched in hydrophobic residues (often flanked by positively charged amino acids) [19] that are typically exposed by proteins in non-native conformations. Reversible binding of hydrophobic peptides to the C-terminal substrate-binding domain (SBD) of Hsp70 is regulated by ATP binding and hydrolysis at the nucleotide-binding domain (NBD) (Fig. 2A). The ATP-driven conformational cycle of Hsp70 is coordinated by Hsp40-class J-domain proteins (DnaJ in bacteria) and nucleotide exchange factors (NEFs; GrpE in bacteria) [20]. Hsp40 delivers substrates to the open, ATP-bound state of Hsp70 (Fig. 2B, state II). Binding of Hsp40 and substrate protein synergistically triggers the hydrolysis of bound ATP [21], thereby generating a stable complex between the substrate protein and Hsp70 in the closed, ADP-bound conformation [22] (Fig. 2). Subsequent NEF-binding catalyzes ADP/ATP exchange and facilitates substrate release, with the resulting folding intermediate progressing either directly to the native

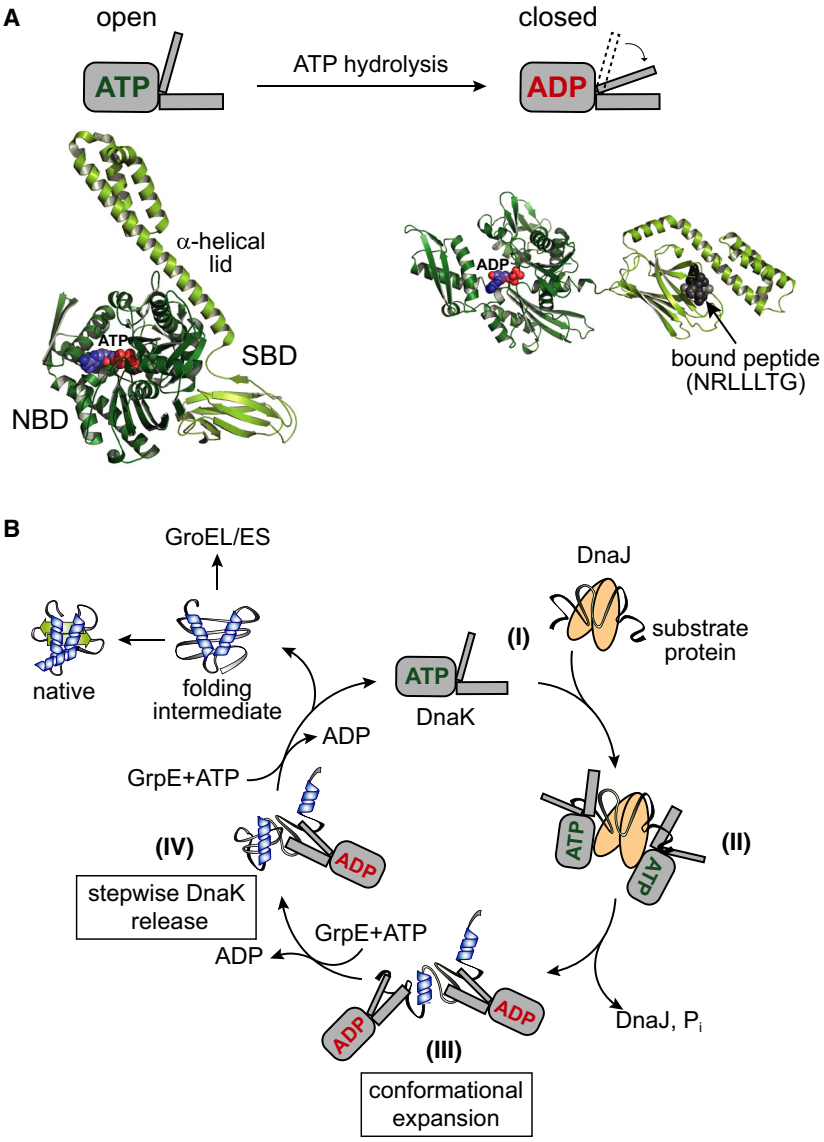

Fig. 2. Structure and reaction cycle of the Hsp70 chaperone system. (A) Structure of the bacterial Hsp70, DnaK. ATP binding to the NBD stabilizes the open state of DnaK (left; PDB 4B9Q) [104], in which the α-helical lid of the SBD is associated with the NBD. Upon hydrolysis of ATP to ADP, Hsp70 transitions from the open state with high on- and off-rates for peptide substrate, to the closed state (right; PDB 2KHO) [105], in which NBD and SBD are separated and the α-helical lid is closed over the peptide-binding cleft (low on- and off-rates for peptide substrate). Bound nucleotide (ATP or ADP) and bound model peptide (sequence NRLLLTG) are shown in space-filling representation. (B) Reaction cycle of the bacterial Hsp70 system. A non-native protein is captured by Hsp40 (DnaJ) dimer and delivered to ATP-bound DnaK (state I), leading to a transient ternary complex between the DanJ, DnaK: ATP and substrate (state II). Interaction with DnaJ triggers ATP hydrolysis on DnaK, generating the closed state and stabilizing an expanded conformation of the substrate in complex with DnaK:ADP (state III). Multiple copies of DnaK may be bound simultaneously. ADP release catalyzed by the nucleotide exchange factor GrpE, and rebinding of ATP, triggers substrate release for folding or possible transfer to downstream chaperones, such as GroEL. Note that nucleotide exchange may not occur simultaneously in all Hsp70 molecules, resulting in a stepwise substrate release (state IV). Figure modified from Ref. [9].

state or being transferred to other chaperone systems, such as a chaperonin or Hsp90 [23,24] (Fig. 2B, state III). The structure and function of Hsp70 and its cofactors have recently been reviewed [16–18]. Here, we focus on ways in which the functional cycle of Hsp70 can be leveraged to accelerate client protein folding.

A fundamental function of the Hsp70 chaperone system is to inhibit protein aggregation, thereby indirectly facilitating (re)folding *via* kinetic partitioning [25]. In this model, binding to Hsp70 prevents aggregation by shielding hydrophobic regions in non-native proteins, and efficient folding upon Hsp70 release occurs when the folding rate constant is higher than the rate of rebinding to Hsp70. Aggregation remains suppressed as long as rebinding of folding intermediates is faster than aggregation. However, this basic function of aggregation prevention does not account for an additional important activity of chaperones: their ability to accelerate folding beyond the folding rate observed in the absence of aggregation (such as under single-molecule conditions). Notably, recent work has revealed that the Hsp70 system can also accelerate the folding of the model multidomain protein firefly luciferase (FLuc) up to ~ 20-fold [26]. Importantly, in these experiments folding was studied under conditions that excluded aggregation, allowing a comparison of folding rates with and without chaperones.

How does the Hsp70 system catalyze folding? Accumulated evidence suggests that two complementary activities are involved: unfolding of misfolded states by

Hsp70 binding, and biasing of the folding pathway toward a fast trajectory initiated from the Hsp70-bound state. ATP-driven unfolding of substrate proteins by the Hsp70 chaperone system has been demonstrated based on protease susceptibility [27], measurements by nuclear magnetic resonance (NMR) spectroscopy [28], and hydrogen/deuterium exchange–mass spectrometry (H/DX-MS) [26,29]. Fluorescence-resonance energy transfer experiments further support unfolding as evidenced by extreme conformational expansion of the substrate protein [26,30,31]. The observed expansion has been attributed to steric repulsion arising from the binding of multiple Hsp70 molecules, driven by the free energy of ATP hydrolysis [32] (Fig. 2B, state III). This 'unfoldase' activity allows the chaperone system to resolve kinetically trapped, misfolded states, but does not fully explain the function of Hsp70 in accelerated folding.

The experiments with FLuc showed that folding initiating from the Hsp70-bound state was kinetically more efficient – for a fraction of molecules – than folding from denaturant, implying that the chaperone shaped the folding pathway [26]. We propose two non-mutually exclusive explanations for this experimental result. First, Hsp70 may allow acquisition of, and stabilize, partial (native-like) structure in the bound state prior to substrate release, thereby hastening subsequent folding (Fig. 2B, state III). Consistent with this possibility, transient secondary structure has been detected by NMR in DnaK-bound hTRF1, a 53-residue model client [33], and H/DX-MS experiments suggested residual structure in DnaK-bound FLuc [26]. Indeed, residual structure in the denatured state ensemble [34,35] can substantially influence the pathway and outcome of folding [36–38].

A second possible mechanism for accelerated folding is that stochastic, asynchronous release of Hsp70 molecules from the substrate protein prevents simultaneous collapse and misfolding of regions of the polypeptide chain that form separate domains in the native state (Fig. 2B, state IV). This hypothesis is supported by pulsed-label H/DX-MS of FLuc folding [26] and is consistent with NMR analyses of hTRF1 [39,40]. hTRF1 can bind between one to three DnaK (Hsp70) molecules, which would result in conformational heterogeneity at the onset of folding, providing access to alternative folding trajectories that may be poorly sampled in the absence of the chaperone.

Is this folding mechanism general for Hsp70 substrates? Hsp70 chaperones interact with a substantial fraction of the proteome (~ 30%) in bacteria and eukaryotes [41,42]. Thus, the mechanism described above might accelerate the folding of many client proteins, in particular those that populate stably misfolding intermediates (see 'Folding problems and chaperone solutions' below). Studies of Hsp70 function have so far been restricted to a relatively small number of model proteins. Going forward it will be important to study a broader range of substrates, including endogenous clients of the chaperone and especially those comprising multiple domains. It also formally remains to be established whether the eukaryotic Hsp70 machinery can catalyze folding reactions. Eukaryotes use a large number (> 40) of diverse J-proteins to tune the substrate specificity of Hsp70s [43,44], which may also modulate the function of the chaperone in accelerating folding.

Beyond *de novo* folding, the concept of accelerated folding by Hsp70 has important implications for protein homeostasis. Recent work has shown that Hsp70 plays a critical role in stabilizing heat-labile proteins against thermal denaturation in *Escherichia coli* [45], and to maintain the native state of such proteins *in vitro* at the expense of ATP hydrolysis, even under conditions that would otherwise be denaturing [26,46]. By resolving kinetically trapped, misfolded states that are populated during stress-induced unfolding and by accelerating their refolding, the Hsp70 system effectively remodels the energy landscape in favor of the native state.

Catalysis of folding by the GroEL/ES chaperonin

Chaperonins (also referred to as Hsp60s) are large oligomeric complexes that function as nanocages for single protein molecules to fold in isolation [47–51]. They participate in folding ~ 10% of the cytosolic proteome, including essential proteins that fail to reach their native state spontaneously and cannot utilize other chaperone systems [52–56]. The bacterial chaperonin GroEL consists of two rings of seven identical ~ 60 kDa subunits, stacked back-to-back. Each subunit comprises an equatorial ATPase domain, an intermediate hinge domain, and an apical domain that exposes hydrophobic residues for binding non-native substrates (Fig. 3A). The folding chamber is created by interaction with GroES, a lid-shaped heptamer of ~ 10 kDa subunits that binds to the apical domains of GroEL (Fig. 3A).

The two rings of GroEL function sequentially as folding chambers regulated allosterically by the GroEL ATPase [47,51] (Fig. 3B). Non-native substrates are captured by interaction with multiple apical domains of GroEL. Binding of ATP and GroES then displaces

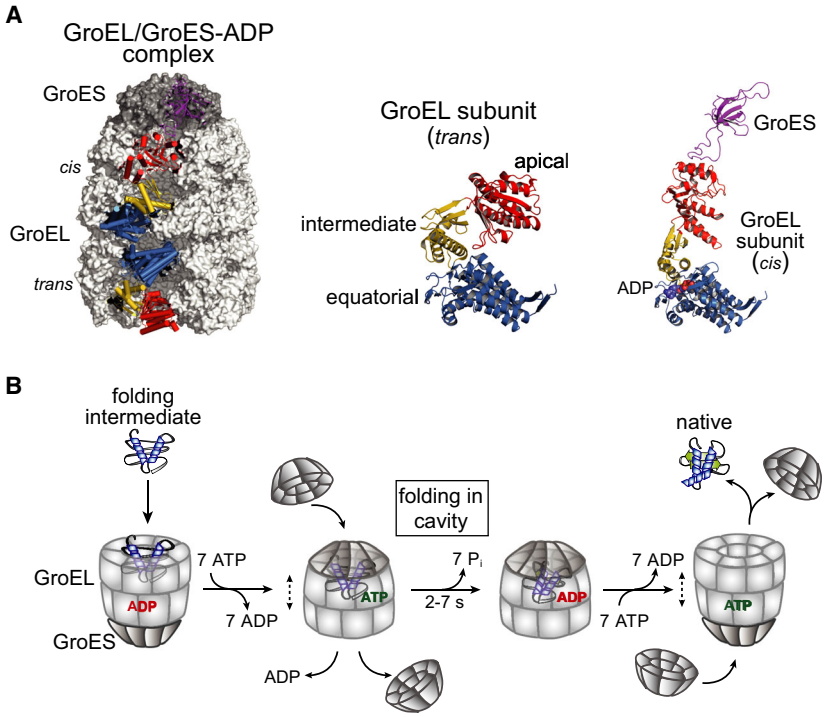

Fig. 3. Structure and reaction cycle of the GroEL/ES chaperonin. (A) Left, crystal structure of the asymmetrical GroEL:ADP:GroES complex (PDB 1AON) [58] in space-filling representation. One subunit in each GroEL ring is displayed schematically, with the equatorial domain in blue, the intermediate domain in yellow, and the apical domain in red, the interacting subunit of GroES is shown in magenta. Middle, conformation of the GroEL subunit in the open state *trans*-ring. Right, conformation of the GroEL subunit in the GroES-bound state (*cis*-ring) in ribbon representation. (B) Reaction cycle of GroEL/ES. Substrate protein binds to the *trans*-ring of the GroEL:ADP:GroES complex, followed by encapsulation upon ATP-dependent GroES binding. This step is accompanied by transient separation (dashed double-arrow) and subsequent reassembly of the GroEL rings. The protein is free to fold within the chaperonin nanocage for the time required to hydrolyze the 7 ATPs in the GroEL *cis*-ring. ATP binding to the *trans*-ring then triggers release of ADP and GroES, allowing folded protein to exit, completing the cycle. Incompletely folded protein is rapidly recaptured by GroEL. Figure modified from Ref. [9].

the substrate into a cavity capped by GroES (the *cis*-ring) (Fig. 3B). Due to a negative allosteric coupling of the rings [51], this step is accompanied (*in vitro*) by transient separation of the GroEL rings [57]. Extensive conformational changes enlarge the *cis*-ring cavity and alter the physical properties of its inner surface from hydrophobic to hydrophilic [58]. The negative allostery between rings facilitates substrate release from the *trans*-ring and disfavors the formation of symmetric complexes with both rings being GroES-capped, which are compromised in substrate capture [57]. Following encapsulation, proteins up to ~ 60 kDa in size are allowed to fold for the time it takes the GroEL *cis*-ring to hydrolyze its 7 ATPs to ADP (~ 2–7 s dependent on temperature) [59] (Fig. 3B). Binding of ATP to the *trans*-ring then induces an allosteric signal that causes ADP and GroES to dissociate from the *cis*-ring. Folded protein is released, while incompletely folded or misfolded molecules may rapidly rebind for another folding cycle. During cycling, the protein spends most of its time (> 80%) in the encapsulated state where folding occurs [59]. Some aspects of GroEL/ES function, such as the relative importance of symmetric and asymmetric complexes, are a matter of ongoing research (reviewed in Ref. [47]). Here, we focus our discussion on recent work that has advanced the concept that GroEL/ES is a catalyst of protein folding.

It is well established that by encapsulating single protein molecules in its central cavity, GroEL/ES allows folding to proceed unimpaired by aggregation. Work over the past two decades has moved our understanding beyond this fundamental principle and demonstrated that the chaperonin nanocage represents a privileged folding environment in which formation of kinetically trapped intermediates that would otherwise slow or halt spontaneous folding is avoided [55,60–62]. As a result, GroEL/ES provides kinetic assistance to the folding process and accelerates the folding of various proteins ~ 20- to 100-fold above their spontaneous folding rate. These include

destabilized variants of maltose-binding protein (MBP) [59,60,63–65], *Rhodospirillum rubrum* Rubisco [66,67], bacterial proteins with topological knots [68], the *E. coli* prolidase enzyme PepQ [62], and several *E. coli* proteins with a (βα)8 TIM-barrel fold [55,61]. In all cases, the slower rate of spontaneous folding was not due to transient aggregation, implying that the chaperonin altered the folding energy landscape for these substrates.

How does GroEL/ES catalyze protein folding? The following features of the chaperonin system have been implicated in accelerating folding: (a) unfolding of substrate protein upon binding and ATP-dependent apical domain movements [67,69,70]; (b) the net negative charge of minus 42 of the *cis*-cavity GroEL wall [55,59,60,63,64]; (c) the volume of the folding chamber relative to the size of the encapsulated substrate [63,64]; (d) and the dynamic C-terminal extensions that extend from the equatorial domains of each subunit into the central cavity [62,64,71]. The relative contribution of these factors may be substrate-dependent.

In the context of the chaperonin reaction cycle, the distinctive structural features of GroEL/ES implicate several nonmutually exclusive mechanisms in folding catalysis. Prior to encapsulation, stretching of bound substrate by ATP-mediated apical domain movements may prime the substrate for efficient folding upon encapsulation [67,69,70]. This step would also occur upon substrate rebinding in consecutive chaperonin cycles, but was found to be dispensable for accelerated folding of mutant MBP [70]. Moreover, folding is also accelerated upon stable protein encapsulation without GroES cycling [59,61,64,66].

Steric confinement in the GroEL/ES cavity is predicted to smooth the folding energy landscape by restricting the conformational freedom of the encapsulated substrate [72–74]. In support of this idea, photoinduced electron transfer/fluorescence correlation spectroscopy and H/DX-MS experiments have demonstrated reduced chain mobility of mutant MBP upon encapsulation, facilitating native interactions [59,65]. Additionally, engineered intramolecular disulfide bonds that mimic the confinement effect have been shown to accelerate spontaneous folding of MBP to the degree achieved by GroEL/ES, with no further rate acceleration upon encapsulation of the disulfide-bonded protein [60]. Encapsulation promotes segmental acquisition of structure in the TIM-barrel core of DapA [61] and allows MetF to fold into an oligomerization-competent monomer that does not otherwise form in free solution, even in the absence of aggregation [55]. Folding enhancement by confinement is likely to be most significant for proteins that populate conformationally dynamic intermediates (see 'Folding problems and chaperone solutions' below).

During folding, the encapsulated substrate may additionally be remodeled by hydrophobic interactions with the disordered C-terminal tails of GroEL, which contain the conserved repeat motif Gly-Gly-Met [62,64,71]. While these sequences have also been implicated in substrate binding [75,76], how exactly they modulate folding remains to be determined. The highly charged character of the GroEL/ES *cis*-cavity was found to be critical in restricting chain mobility of encapsulated protein [59] and has been proposed to promote hydrophobic compaction by inducing ordered structure in water molecules associated with the cavity wall [77]. However, experimental evidence for the existence of cavity-confined water is still lacking [78].

Although catalysis of folding by GroEL/ES has so far been observed for a relatively small set of proteins, it is striking that the folding of obligate, endogenous substrates of the chaperonin is most strongly accelerated. Some of these proteins, sharing the TIM barrel topology, tend to fold in just a few cycles of chaperone action, implying that coevolution of substrate and chaperonin has optimized the *in vivo* folding rate. Such mutual adaptations would be limited by the intrinsic folding properties of a specific substrate, and by the fact that the chaperonin must be able to fold numerous different substrates [79]. Proteins with the TIM-barrel fold, which form a large group of topologically similar substrates of GroEL/ES in *E. coli* [52,54,80], may have been more successful in optimizing their chaperonin-assisted folding than proteins with less frequent topologies. Further studies on endogenous substrates with different topologies will be required to establish general principles underlying the function of GroEL/ES as a folding catalyst.

Folding problems and chaperone solutions

Protein folding is slowed by energy barriers that separate folding intermediates from the native state (Fig. 1). Broadly, intermediates can be characterized as either stably misfolded or conformationally dynamic. Both classes of intermediate bury hydrophobic surface and are therefore stabilized by high solvent entropy relative to the unfolded state. However, because folding intermediates are only marginally stable, other forces can tip the balance to influence the rate of folding. Misfolded intermediates are characterized by long-lived, non-native main- and side-chain interactions (hydrogen bonding, Van der Waals contacts and electrostatic interactions) that are

enthalpically favorable. In contrast, intermediates that are dynamic relative to the native state have limited stable structure (native or non-native) and their stability is enhanced by their high configurational entropy. Recent analysis of chaperone-catalyzed folding suggests that the Hsp70 system preferentially attends to proteins that populate misfolded states while the GroEL/ES chaperonin system promotes folding of proteins that tend to populate conformationally dynamic folding intermediates (Fig. 4).

Interdomain misfolding is thought to be a widespread cause of slow folding and would be especially prevalent in proteins with large domain–domain interfaces [81–83]. Research on the model protein FLuc has advanced our understanding on how the Hsp70 system resolves such misfolded states. FLuc spontaneously misfolds upon stress-induced unfolding, with non-native interactions between the subdomains of its large N-terminal domain frustrating subsequent refolding [26,84,85]. As described above, Hsp70 cooperates with its cochaperones to both unfold these misfolded intermediates and smooth the energy landscape of subsequent folding. Interestingly, this Hsp70 mechanism is highly efficient during cotranslational folding of FLuc,

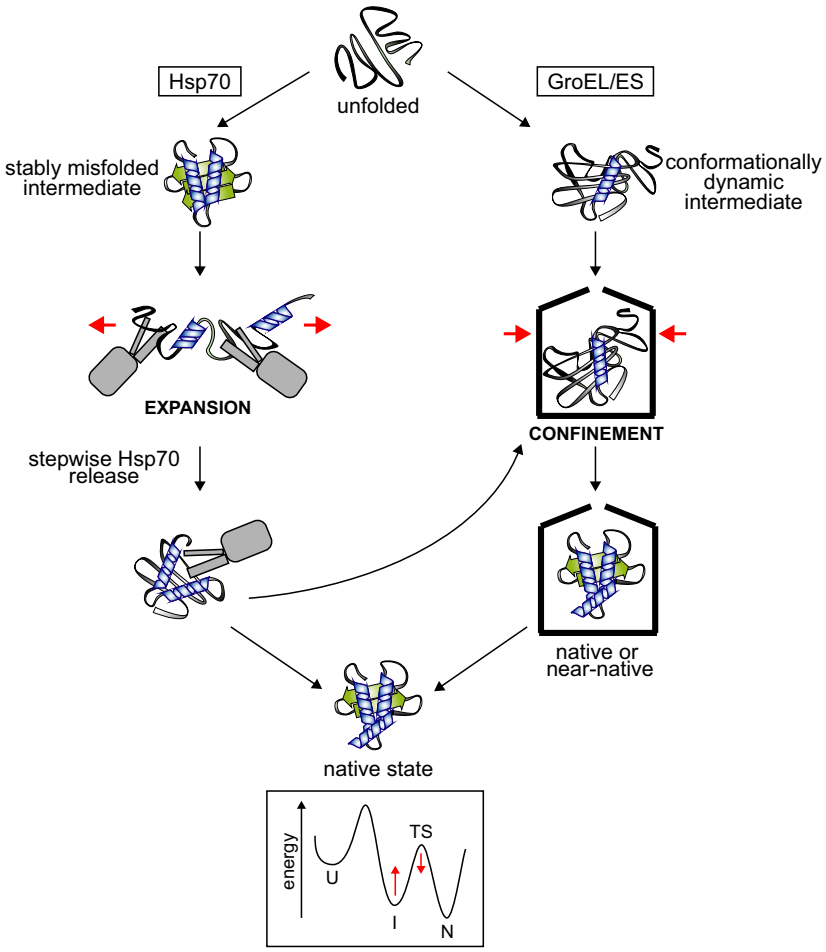

Fig. 4. Function of Hsp70 and GroEL/ES in accelerating folding. The Hsp70 and GroEL/ES chaperone systems attend to protein subsets that populate different types of kinetically trapped folding intermediates. Stably misfolded intermediates (left) are resolved by conformational expansion, driven by the ATP-hydrolysis-dependent binding of multiple Hsp70 molecules. Additional features of the Hsp70 system, such as stabilization of native-like secondary structure in the bound substrate protein and/or stepwise release of Hsp70 molecules, bias subsequent folding to a fast trajectory for a fraction of molecules. Intermediates that are conformationally dynamic (right) are instead destabilized by confinement in the GroEL/ES cavity. C-terminal extensions of GroEL protruding into the cavity contribute to accelerated folding. Proteins that fail to fold rapidly with assistance by Hsp70, which functions upstream in the folding pathway, can partition to GroEL/ES. The effect of chaperones on the folding free-energy landscape is illustrated in the lower panel. For both Hsp70 and GroEL/ES, selective acceleration of the folding reaction is realized by destabilization of intermediate states (I) relative to the transition state (TS), without altering the free energy of the native state (N).

facilitating sequential folding of the N-terminal subdomains as they emerge from the ribosome [86,87]. The Hsp70 chaperone system would also correct cotranslational misfolding when domain folding at the ribosome does not synchronize with translation rate [88–91].

Consistent with Hsp70 and GroEL catering to proteins having different folding problems, the Hsp70 system cannot deal with the obligate substrates of GroEL/ES. Although aggregation of these proteins is inhibited by cycles of Hsp70 binding and release, folding is very inefficient, if it occurs at all [52,55,61,64]. What distinguishes chaperonin substrates from those of Hsp70? Proteins that depend on GroEL/ES to fold are primarily 35–60 kDa in size, consistent with the volume of the chaperonin cavity, and typically have α/β and α+β domain topologies that are stabilized by long-range interactions [52,54,80,92]. Proteins with large domains and high topological complexity are likely to undergo indiscriminate hydrophobic collapse at early stages of the folding reaction. The resulting intermediates tend to be stabilized by high solvent and configurational entropy. Theory predicts that for domains ≥ 200 amino acids, the hydrophobic forces are no longer sufficient to reduce the effective conformational space to a size that allows folding at a biologically relevant time scale [93]. Confinement in the GroEL/ES cavity uniquely addresses this particular cause of slow folding by lowering the entropic component of the folding energy barrier and reducing the search time for native contacts [59–61] (Fig. 4).

In some cases, the kinetic trap may be so deep that spontaneous folding is essentially undetectable under standard *in vitro* conditions, even in the absence of aggregation. This phenomenon has been observed for the GroEL-substrate MetF and for actin, a major obligate substrate of the eukaryotic chaperonin TRiC [55,56]. TRiC provides steric information through chaperonin subunit-specific interactions that direct the folding of actin [56,94]. We speculate that extreme dependence on (specific) chaperones for folding is a consequence of coevolution of chaperone and substrate. In these cases, the sequence space of the substrate protein may also be constrained by obligate cofactor binding (as in MetF), or extensive functionally critical protein–protein interactions (actin).

How are the different chaperone activities in the bacterial cytosol coordinated into a functional network? GroEL acts downstream of the more abundant, general cytosolic chaperones trigger factor and Hsp70 [23,41,95–97]. Proteins that do not fold efficiently with the upstream chaperones are maintained by Hsp70 in a soluble state competent for folding upon transfer to GroEL, such that the network functions as a 'selective percolator'. It is also possible that optimal folding of some bacterial proteins requires sequential processing by multiple chaperone systems. Indeed, a subset of *E. coli* proteins were shown to require the combined action of trigger factor, the Hsp70 system and GroEL/ES for maximum solubility in a reconstituted system [98]. Conceivably, resolution of misfolded intermediates by Hsp70 could generate dynamic states that are primed for accelerated folding by GroEL/ES (Fig. 4). Likewise, under certain conditions the refolding yield of an Hsp70 substrate can be enhanced by cooperation of Hsp70 with the chaperone Hsp90 [24]. Direct physical interactions between chaperones may enhance the efficiency of the network and favor sequential processing of some substrates [99].

Conclusions and perspectives

Accumulated evidence has now shown that molecular chaperones can shape the energy landscapes of protein folding to accelerate folding reactions. This observation emphasizes the fact that not only the yield, but also the rate of folding is critical *in vivo*. Optimally, protein biogenesis is rate-limited by protein synthesis. Slow folding proteins are at risk of aggregation or premature degradation, and it is our view that catalysis of protein folding by chaperones is a vital function that harmonizes folding speed with the rate of translation. Recent work has begun to illuminate the fascinating mechanisms by which chaperones stimulate folding. A key finding is that the major chaperone systems of the bacterial cytosol attend to different categories of folding problem: The Hsp70 system catalyzes the folding of stably misfolded species, while confinement in GroEL/ES accelerates the conversion of conformationally dynamic intermediates to the native state. Although technically challenging, further insight will come from mapping the conformational progression during folding for a greater variety of authentic *in vivo* chaperone clients.

Besides Hsp70 and the chaperonins, other chaperone systems offer additional solutions to distinct folding problems. Eukaryotic Hsp90, for example, and its cochaperones play a critical role in the conformational maturation of specific clients such as protein kinases, stabilizing metastable states that are poorly populated in the absence of these chaperones [100]. Furthermore, ATP-independent chaperones such as small heat shock proteins, trigger factor, and Spy (in the bacterial periplasm) have been shown to modulate protein folding pathways, although whether and how these chaperones accelerate the folding of endogenous substrates is at present unclear [101–103]. Finally, it will be important

to recapitulate the full complexity of folding *in vivo*, by studying chaperone action also in the context of translation. For instance, the ribosome has been shown to directly modulate protein folding [11] and may thus dictate how chaperones interact with nascent proteins [12].

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