Protein folding mediated by trigger factor and Hsp70: new insights from single-molecule approaches

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Chaperones assist in protein folding - but what this common phrase means in concrete terms has remained surprisingly poorly understood. We can readily measure chaperone binding to unfolded proteins, but how they bind and affect proteins along folding trajectories has remained obscure. Here we review recent efforts by our labs and others that are beginning to pry into this issue, with a focus on the chaperones trigger factor and Hsp70. Single-molecule methods are central, as they allow the step-wise process of folding to be followed directly. First results have already revealed contrasts with longstanding paradigms: rather than acting only 'early' by stabilizing unfolded chain segments, these chaperones can bind and stabilize partially folded structures as they grow to their native state. The findings suggest a fundamental redefinition of the protein folding problem, and a more extensive functional repertoire of chaperones than previously assumed.

Introduction

It is difficult to overstate the importance of the Hsp70 chaperone system. Preserved across all domains of life it is essential to an organism's survival during thermal stress [1, 2]. Hsp70 is also central to normal cellular physiology, and is for instance involved in protein synthesis and degradation [3-6], in trans-membrane translocation of proteins [7, 8], and in modulating the activity of key receptors and kinases [9, 10]. In these many roles, Hsp70 is supported by a host of co-chaperones such as J-domain proteins [9]. The prokaryotic chaperone trigger factor (TF) is the second general chaperone system in E. coli, involved in aggregation prevention of hundreds of proteins and cotranslational assistance [11]. TF is believed to act before the onset of protein folding generally, as it interacts with the ribosome and the nascent chain during mRNA translation [12-14]. TF directly binds the L23 large ribosomal protein close to the ribosomal exit tunnel [12], and can continue binding the newly synthesised chain after dissociation from the ribosome [15]. Simultaneous deletion of DnaK and TF causes aggregation of a large number of proteins and is synthetically lethal in *E. coli* at temperature above 30°C, while overexpression of the chaperones SecB or GroEL can partially restore cell function [16-21].

Numerous structural and biochemical studies over the last five decades have elucidated key modes of action of the Hsp70 and TF chaperone systems [2, 22-40]. Hsp70 is believed to perform its diverse physiological roles by binding and releasing unfolded peptide segments, which may extend from different protein conformers including aggregates, in an ATP-driven cycle [9]. Trigger factor is thought to stabilize nascent chains in their unfolded state, thus fulfilling a classic holdase function [41, 42]. However, current techniques are not well suited to

detect the conformational dynamics of chaperones and their substrates, which are central to protein folding and possible chaperone control. Key complications are the dynamical nature of folding and partially folded states, averaging effects within populations, as well as transient or permanent aggregation between substrates (Fig. 1) [43]. Single-molecule techniques have the potential to fill in this gap and resolve differences between various substrate folding states that may be crucial to understanding chaperone mechanisms and physiological roles [44, 45].

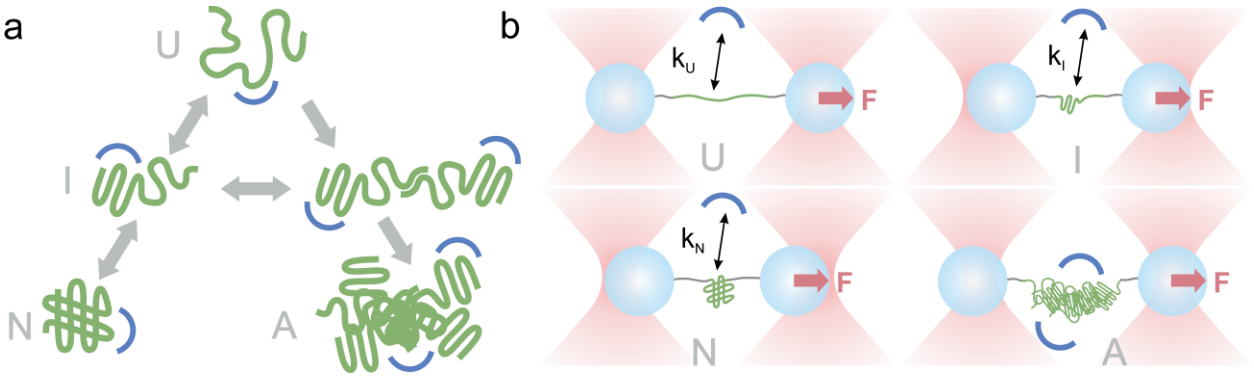

Fig. 1. Untangling folding and aggregation pathways with optical tweezers. (a) Cartoon of a folding pathway from unfolded (U) via intermediates (I) to the natively folded states (N). Averaging in ensemble studies hide transiently visited folding intermediates, as well as the binding of chaperone (blue) and the protein conformational changes they may mediate. Reversible association and irreversible aggregation between folding intermediates [46] can obscure whether chaperones affect folding or aggregation. (b) Single-molecule methods such as optical tweezers can isolate individual substrate states, and hence probe how chaperones affect them in the absence of confounding interactions with other substrate molecules. Direct measurement of tension (F) on the tethered substrate allows one to study chaperone-mediated stabilization and conformational changes. Using constructs of multiple N-to C-terminally linked protein, one can investigate aggregation between proteins and how chaperones affect it (bottom-right) [44].

In this review we focus on investigations of Hsp70 and TF by optical tweezers. Hsp70 was shown to use its ATP-driven clamp-like lid to capture and tightly stabilize folded protein structures, which updates the decades old canonical model of Hsp70 function [47]. This new

mode of action promotes folded rather than unfolded states, acts during rather than before folding, and utilizes the Hsp70 lid rather than the groove as main substrate binding device. Investigation of TF revealed that it too remains associated during later phases of folding. By transiently binding partially folded states, TF can limit non-native interactions between distant sites along a protein's sequence. TF thus does not solely act as a holdase but assumes a more active role as a foldase [48]. This 'open cavity' mechanism could apply more generally to other chaperones. Finally, we discuss the new questions raised by these results, focussing also on the role of chaperones in co-translational folding.

Extending the Hsp70 canonical model

The last decades have produced invaluable insights into Hsp70-substrate interactions and established its role as cell-regulatory hub [11]. Experimental structures revealed the complex between Hsp70 constructs and a small peptide, with the latter bound to a groove in the substrate binding domain of Hsp70s and covered by a helical lid (Fig. 2A-B) [22, 24, 25, 49-51]. Hydrophobic residues were found to be common among Hsp70-interacting peptides, with flanking positive residues that increase affinity [26, 33, 52-55]. The ATP hydrolysis cycle was known to promote peptide binding and release by closing and opening the lid and the peptide binding groove [25, 31, 51, 56]. The resulting canonical model thus suggests that peptide segments extending from diverse protein conformers are stabilized, through a binding-and-release cycle that is under nucleotide and co-chaperone control (Fig. 2G) [57]. The many cellular roles of Hsp70, which include folding-assistance, disaggregation, translocation, and regulation

of the activity of native proteins, have been interpreted and explained at the molecular level within the context of this simple principle. For instance, the binding of exposed peptide segments by Hsp70 could protect nascent chains against aggregation and stabilize partially unfolded, inactive conformations of steroid hormone receptors.

The complexity of the Hsp70 (DnaK in bacteria) system makes it a challenging target for single-molecule investigations. Not only the chaperone itself, but also its protein substrates undergo extensive conformational changes as they fold, while interactions between chaperone and substrate are short-lived, and co-chaperones play important additional roles. The latter include the J-protein (DnaJ) and nucleotide exchange factor (NEF; GrpE) co-chaperones, which help recruiting Hsp70 and controlling the ATP cycle. Hence, a bottom-up approach was followed in [47]: substrate conformations were controlled mechanically using optical tweezers, Hsp70 conformations were controlled by arresting the ATP cycle at different steps, chaperones and co-chaperones were added one-by-one, and finally Hsp70 sub-domains were mutated or deleted to assess their roles.

A screening of the different Hsp70 states revealed the predominantly closed-lid ADP-state to be the most deviating from previously established models. Unfolded maltose binding protein (MBP) substrates were stabilized in that extended state by exposure to Hsp70-ADP, as evidenced by an inability to *refold* upon relaxation to zero tension. This result is fully consistent with the canonical model, which also dictates stable peptide binding in the ADP state, as well as with more recent single-molecule FRET studies [58]. Surprisingly, however, nearly completely refolded MBP substrates were also stabilized by Hsp70-ADP, now evidenced by an inability to

unfold up to 65 pN as the technical maximum that can be measured in this assay (Fig. 2 D-F). The stabilization was specific: In APO and ATP states, Hsp70 did not stabilize folds, consistent with release. Fully folded native states were left unaffected [47], while just peeling-off a few C-terminal alpha helices from the main core structure was sufficient to trigger the stabilizing effect. Paradoxically, these Hsp70 modes thus act in different directions, with one promoting folded states and the other unfolded states (Fig. 2G).

These findings raised numerous questions. For instance, why was this binding mode not observed before during decades of *in-vitro* study? First, partial folding intermediates that could be recognized by Hsp70 are usually not stable and hence cannot be isolated for use in bulk assays. In addition, methods that monitor folded states of proteins, like circular dichroism spectroscopy, tryptophan fluorescence, and NMR require rather high protein concentrations. This is often not a problem for natively folded proteins but folding intermediates or misfolded proteins then tend to aggregate. Another key question is how stabilization is achieved at the molecular level. Do these folded structures fit under the lid, in a clamp-like fashion (Fig. 2C)? Arguing for this option, the lid can transiently open in the ADP-state [59], and remain open when interacting with folded proteins [59, 60]. Consistently, a lid-truncation abolished the mechanical stabilization [47]. Mutating the groove diminished it, suggesting cooperative binding of the Hsp70 groove to peptide segments and lid to nearby folded states [47]. However, this issue remains incompletely resolved until complex structures can be determined directly. Novel techniques for obtaining structural details of these dynamic complexes are getting within reach [61]. Simulations have been used for a similar purpose [62, 63] while selective labeling

schemes in NMR and recent advances in cryo-EM open a path to handling ever-larger partially dynamic complexes [64-67].

Another question is whether non-mechanical perturbations can also trigger the stabilization of folded structures. One may consider investigating this issue with moderate temperature increases, to partially unfold the substrate while keeping the chaperone intact. However, native MBP unfolds at higher temperature than Hsp70 [68, 69]. The *E. coli* protein RepE54 (F-plasmid replication initiation protein) appeared more promising, given its suggested lower unfolding temperatures and known interaction with Hsp70. RepE54 was indeed found to unfold at higher temperatures when Hsp70-ADP was added, as detected using fluorescence measurements of endogenous tryptophans in MBP [47] (Fig. 2H). This upshift was not seen for lid-truncated Hsp70, consistent with its importance in stabilizing folds. Such thermodynamic stabilization could allow cells to maintain thermo-labile proteins close to their native state during heat stress, when ATP levels decrease and ADP levels rise. It would be of interest to determine what type of patches on the substrate surface Hsp70 recognizes, and hence how selective Hsp70 is in this mode.

The notion that Hsp70 can bind surfaces of tertiary protein structures helps refine our understanding of Hsp70-mediated disaggregation. Together with co-chaperones that include the J-proteins, Hsp70 can ‘re-solubilize’ aggregates, including fibrillar amyloids linked to neurodegeneration [38, 39, 70-74]. On first sight, disaggregation and related unfolding [37] by Hsp70 may seem in opposition to the observed stabilization of folded structures. However, the stabilization is observed in the ADP state and hence transient. Moreover, stabilizing partial folds

also limits the formation of more stable structures such as larger folds or aggregates (Fig. 2I). Overall structural stability can thus be lower. As noted previously, peptide segments that are extended and exposed long enough to bind Hsp70 before they fold or aggregate, are stabilized in their unfolded state. They may thus progressively dissociate from aggregates by entropic pulling [36, 75, 76]. Hsp70s binding to individual misfolded protein monomers may help them unfold through the same mechanism [36]. One also cannot exclude that Hsp70 directly destabilizes specifically misfolded structures, for instance by transiently competing for non-native interfaces that are part of misfolds, but absent in native conformations. How the stabilizing and destabilizing impact of Hsp70 is regulated remains unclear, and may depend on substrate sequence and conformation, as well as on co-chaperones that can target specific conformations and modulate the ATP cycle.

The binding of folded structures provides a new perspective on the foldase function of Hsp70. It indicates that Hsp70 can affect folding pathways until the folding substrates are nearly native. Hsp70 is typically thought to act early during folding on peptide segments, while other chaperones such as Hsp90 and GroEL act late on folded structures. The findings show that Hsp70 can also act late, challenging the idea of a strict hierarchy between Hsp70, Hsp90, and GroEL. How folding trajectories are affected in detail is an open question. Hsp70 could route pathways along specific partially folded states, thus suppressing entry into conformations that may be unproductive for folding. Folding may occur while the protein remains bound to Hsp70 as in the cases of GroEL and the periplasmic chaperone Spy [77], or involve association-dissociation cycles. This active role could complement more passive mechanisms. Specifically, upon release, substrates may find themselves in altered unfolded starting positions within the free energy folding landscape, possibly opening

up new pathways to the native state [78]. Ultra-affinity of Hsp70 for its peptide substrates, driven by high on-rates in the ATP-state and low off-rates in the ADP state, promotes this process [79, 80]. It will be of interest to see whether affinity-modulation by the ATP cycle plays a role in binding to folded structures as well. We note that energy landscapes have traditionally been utilized to describe folding of small isolated proteins in equilibrium, while interactions with Hsp70 and other folding modulators can introduce complex non-equilibrium effects that are far from being understood.

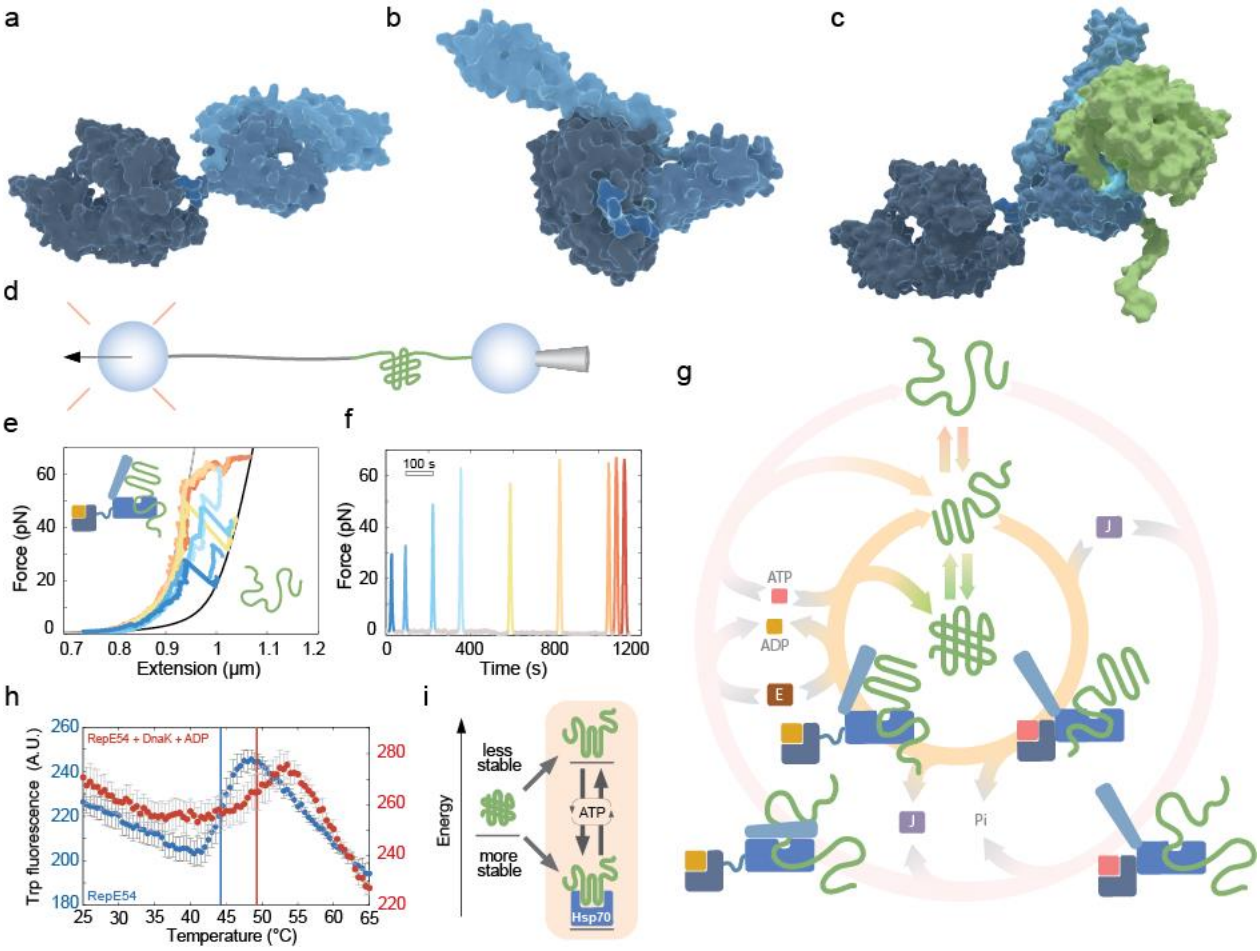

Figure 2. The bacterial Hsp70 chaperone DnaK binds and stabilizes partially-folded states. a) ADP state of DnaK (PDB ID: 2KHO), with the lid predominantly closed over the groove and undocked domains. b) DnaK in the ATP state, with the lid open and domains docked (PDB ID: 4B9Q). c) Hypothetical representation illustrating how partially folded MBP (green, based on PDB ID: 2MV0) could bind DnaK in its ADP state, with the folded part interacting with the lid in an open conformation and the unfolded part binding the DnaK peptide binding groove. Alternatively, the DnaK lid could be in conformations that are more closed or more open. d) Cartoon of the tweezers experiments setup, with MBP tethered between two beads hold by laser trap and micropipette. e) Force–extension curves showing increased stabilization of folded structures, in the presence of DnaK and ADP. Earlier pulls are in blue, later ones in red. f) Corresponding force versus time shows the sequence of pulls on the same MBP molecule and waiting periods at 0 pN. g) The outer red ring represents the canonical Hsp70 model, the inner ring the recent extension. Hsp70 interacts throughout the folding process, from unfolded segments (outer ring) to partially folded structures (inner ring) until the protein is natively folded (center). h) DnaK increases thermal stability of RepE54. Thermal denaturation curves of RepE54 as measured by tryptophan fluorescence in the absence (blue) or presence (red) of tryptophan-free DnaK(W102F) with ADP loading buffer ADP. Vertical lines mark the apparent melting points. Error bars indicate the standard error of the mean over three replicates. i) Hsp70 effect on substrate stability. Hsp70 can transiently stabilize partially folded structures that are less stable than complete folds upon Hsp70 release. Hsp70 binding can suppress the formation of permanently stable aggregates. ATP and cochaperones provide stability control.

Trigger factor as an open cavity foldase

TF appears an unlikely candidate for folding guidance. It is a relatively simple chaperone that does not consume ATP, and is often typified by its binding to the ribosome, where it forms a cradle that accommodates nascent chains emerging from the exit tunnel. TF has long been thought to predominantly protect these polypeptide chains, shielding their hydrophobic segments from unwanted interactions [15], and hence preventing aggregation and protein degradation [42, 81]. Thus, a classic *holdase* function that stabilizes unfolded states [41, 42, 64, 82]. Due to its high cytosolic concentration the majority of TF molecules within the cell are not ribosome bound [83]. TF is known to form dimers, and recently TF has been implicated in the assembly of protein complexes [17]. Its apparent and conditional synthetic lethality with DnaK suggests that it shares some of the functional repertoire of Hsp70 [16-21].

The first single-molecule investigation of TF's effect on the conformation of protein substrates revealed a more involved mode of action than initially thought [48]. The method employed was similar to the one discussed in the previous section. In the absence of chaperone, the C-terminus first detaches from the MBP core upon stretching, followed by unfolding of the core itself at higher force. Important to note here is that in 9% of the experiments, intermediate states were briefly (less than 1s) visited during the large core unfolding transition. These short-lived folded structures are a part of the larger core structure, and hence called partially folded states. Surprisingly, when TF was added, such partially folded states were ‘visited’ more frequently, for longer, and resisted higher forces (Fig. 3A). This stabilization suggests TF directly binds the folded part of the protein chain – not just the unfolded segments. Such direct binding was tested in bulk, by generating MBP truncates that

correspond to predicted partial folds. While full MBP did not bind TF, the truncates did, consistent with the single-molecule results.

Optical-tweezers refolding studies regularly exploit the 'hopping' between different folded states, for instance to extract detailed folding kinetics [84, 85]. Such hops can typically be followed in real-time when the folded structure is comparatively small, as energy costs of the associated contractions are then limited. The MBP structure is comparatively large however, and as a result refolding occurs in obscurity when the force is fully relaxed. There is an important upside however: refolding can be studied without the perturbing applied force, which is how chaperones normally find their substrates in cells. Indeed, partial folds that were stabilized by TF at one moment, subsequently continued to fold and grow in size, when just given some time (order seconds) in the fully relaxed state (Fig. 3A-B). This growth is seen as a reduced measured extension in subsequent stretching. Thus, force here is used to prepare and probe states before and after folding. These data raise intriguing questions about the dynamics of TF-MBP complexes, and suggest that the contacts and conformation of both partners are dynamic enough to allow continued folding.

Another burning question is how stabilizing folds helps folding. One possibility is that by binding and stabilizing domains in multi-domain proteins, TF could limit non-native interactions between them during folding, potentially by more than one TF chaperone [64]. To explore this scenario, four MBP repeats arranged in a head-to-tail manner were stretched and relaxed. Unfolded domains now indeed misfolded and aggregated upon relaxation without chaperone, as evidenced by high unfolding forces (Fig. 3C, bright red trace). With TF present however, the formation of tight misfolds was reduced substantially (Fig. 3C, dark red trace). Thus, TF can limit

non-productive interactions between domains, while still allowing folding interactions within domains (Fig. 3D).

These observations show TF can also act as a *foldase* that promotes native folding. They suggest an 'open cavity' model for TF, in which it provides some of the steric protection of a cavity, while allowing continued substrate dynamics and release, without energy input. The flexible TF appendages that 'wrap around' diverse folded states, as suggested by simulations [86], may explain how TF can act generally on many substrates. It would be of great interest to experimentally resolve such structural features, using for instance NMR [64] or deuterium exchange mass spectrometry [87].

The open cavity model also suggests changes to the folding energy landscape. The binding of TF to (partially) folded structures can stabilize them, thus increasing their lifetime and energy barriers to other states. Binding to folded structures also opens up the possibility of conformational selection, in which certain folded states are favored over others. Note that, in addition to enthalpic contributions, binding can be driven by an overall entropy increase: The liberation of ordered water from the substrate-chaperone binding interface overcompensates the losses in configurational entropy [17, 88]. Importantly, the data suggests why barriers to aggregated states are higher than to native states: the former requires the exposure and hence liberation of at least two domains, whereas the latter involves one domain only. More generally, the suppression of distant interactions along the chain could be a mechanism to distinguish native from non-native contacts at scales below that of domains.

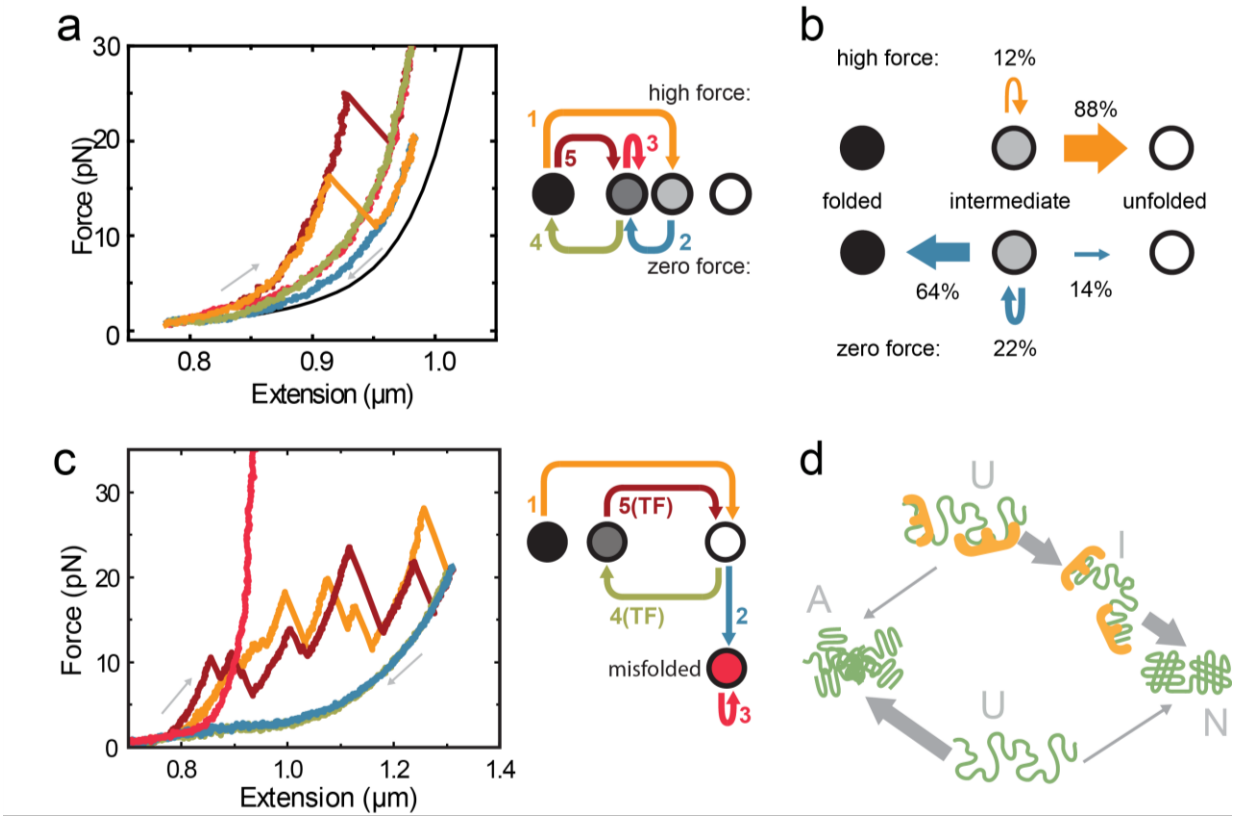

Figure 3. Trigger factor guides early and late protein folding [48]. a) TF-mediated transitions between MBP folded states. Unfolding transitions between fully folded (black circle) to partially folded states (gray circles) that are not observed without TF. These TF-promoted partial folds remain dynamic and can continue growing into more fully folded states, after relaxation in the absence of force. b) Unfolding and refolding probabilities for intermediate partial folds. c) TF suppresses misfolding between domains. Unfolding of protein construct composed of 4 MBP repeats in 4 discrete steps (orange). In the absence of TF, this 4MBP molecule typically misfolds after relaxation (blue) as evidenced by a failure to unfold (bright red). In the presence of TF (dark red), such tight misfolds are absent, some domains fold natively (last step), while others form weaker misfolds (one-before-last step). d) TF (yellow) prevents misfolding or aggregation between domains by suppressing distant interactions along the chain, and hence promoting local interactions.

Cotranslational chaperone action

Proteins are synthesized by ribosomes inside the cell and most of them must fold into complex, specific and stable three-dimensional structures to become functional. Folding of many proteins begins during translation. This co-translational folding imposes various constraints, starting with vectorial synthesis of the protein chain, passage through the narrow ribosomal exit tunnel, subsequent additional interactions with the ribosome [89, 90], as well as with chaperones such as trigger factor (TF) [41].

The synthesis of protein chains is considered to affect and depend on co-translational protein folding [91, 92]. However, the heterogeneous and dynamic folding process of proteins is difficult to follow with bulk methods (Fig. 1). Cotranslational folding is particularly difficult to observe [93]. Single-molecule techniques such as optical tweezers provide a new opportunity to investigate this issue. The mechanism of mRNA translation by ribosomes and nascent chain folding has been the focus of several single-molecule investigations [90, 94-96]. Cotranslational folding was measured recently in a real-time protein biosynthesis study, and it was shown that polypeptide-ribosomal tunnel interactions play a dominant role in the elongation rate during synthesis [97]. These single-molecule studies, however, were performed in the absence of chaperones.

Bioinformatics analyses suggest that about one third of all *E. coli* proteins fold cotranslationally and that translation kinetics influence the folding probability of domains that fold cotranslationally [98]. The rate of translation in *E. coli* is fast, compared to *eukaryotic* translation systems such as that of yeast, with average mRNA translation rates of up to 22 *vs* 5-

9 residues per second at optimal growth conditions [99-101]. Fast *in vivo* protein synthesis can shift the mode of folding of proteins or domains from co- to posttranslational [98]. Vice versa, lowering the rate of synthesis by for example substituting rapidly translated codons to rare, more slowly translated codons [102] can turn some posttranslational folding domains into cotranslationally folding ones [103]. Such kinetic effects are a driving-factor of the meta-stability within the proteome that may lead to misfolding [104]. Interestingly, both too slow and too fast translation can promote misfolding –apparently depending on the level of energetic frustration [105]. Proteins associated with neurodegenerative disorders are often present at cellular concentrations above their maximal solubility. This supersaturation, in combination with reduced chaperone activity in ageing organisms is suggested to be a major contributor to aggregation associated with degenerative diseases [106-108].

It is thought that TF forms a protective extension of the ribosomal tunnel in *E. coli*, and is thus the first chaperone to interact with the nascent chain during translation. TF engages nascent chains once they reached an average length of more than 100 residues [81]. Earlier interactions are with enzymes processing the nascent chain N-terminus, first the peptide deformylase (PDF) followed in many cases by the methionine aminopeptidase (MAP) [109], and for the subset of inner-membrane proteins the signal recognition particle (SRP) [110]. Previous bulk ensemble experiments suggest that TF can unfold polypeptides, which may serve as a mechanism to rescue misfolded proteins co-translationally [42]. During synthesis, some polypeptides may perform initial folding steps already in the distal part of the ribosomal exit tunnel [111]. Cotranslational folding within as well as outside the ribosomal tunnel can exert a pulling force on the nascent chain due to steric exclusion [96, 112]. Similar pulling forces also

occur during membrane translocation [113] and integration [114, 115], and can prevent and even rescue stalling during mRNA translation [96]. Interestingly, TF reduces this force exerted on the nascent chain during protein synthesis [116]. These findings raise the question whether TF counteracts the suppression of stalling events caused by the mechanical force exerted on the nascent polypeptide chain during cotranslational folding.

Mashaghi *et al.* have shown that TF does not solely act as a holdase but assumes a more active role as a foldase, by facilitating local contacts and stabilizing partially folded structures [48]. These experiments investigated posttranslational folding, in the absence of the ribosome. How TF performs additional specifically co-translational roles is an open question. Ribosome-bound TF offers a different functional repertoire than free TF. This is partly due to the fact that TF binding to ribosomal L23 dramatically increases its local concentration favouring low affinity binding and may change possible interaction modes with other proteins for instance [117]. Also, the conformational dynamics of TF differ when bound to the ribosome as opposed to when it is free. Finally, the substrate polymer is exposed to entropic pulling forces due to the confinement of the nascent polypeptide within the ribosomal exit tunnel, whereas in solution there is no such force. The interaction modes of TF may therefore be different when bound to the ribosome.

The DnaK chaperone system acts as a central hub within the chaperone network for newly synthesized proteins, and can also act co-translationally. It has been found to interact with nascent chains during translation and after ribosome dissociation, preventing its aggregation that can result in detrimental neurodegenerative disorders [118, 119]. The N-to C-

terminal vectorial emergence of the nascent chain from the ribosomal tunnel during synthesis allows for the sequential folding of N-terminal segments before later segments are synthesized. This can cause problems during folding of proteins with high contact order, which require native contacts between both ends of the polypeptide [120]. Cotranslational (mis)folding of N-terminal segments could for instance bury N-terminal residues that make vital native interactions with C-terminal segments, and thus prevent native folding. Chaperones such as DnaK and TF both can delay folding and hence prevent such cotranslational misfolding.

The GroEL-GroES chaperonin complex forms a protective cavity that mediates folding of proteins to their native state [121]. The GroELS chaperone system is generally considered to be downstream of the chaperone cascade, since substrates can be transferred from DnaK and TF to GroEL, and hence to act solely post-translationally [122]. Cotranslational interactions of GroELS with nascent chains has been suggested however [123, 124]. The mode of interaction would likely differ from the established GroEL model, since the tethered nascent chain cannot be fully enclosed within GroELS. Several crucial mechanistic questions concerning the interplay between the TF, Hsp70, and GroEL chaperone systems and co-translational folding remain unanswered and invite further study. The Hsp70 system seems to form functional interconnections between TF and GroELS, since proteins accumulate significantly on Hsp70 if TF or GroEL are depleted [11, 124].

Outlook

The ability to zoom in on individual chaperone-substrate complexes opens the door to a host of new and longstanding unanswered questions. One issue is whether TF and Hsp70 remain bound continuously throughout the folding process, and if so, how this affects folding energy landscapes. Whether such interactions can accelerate folding, for instance by topological selection [125-127] or surface annealing [128] have been debated on an abstract level. One may now add another mechanism: promoting folding by binding partially folded states. A related issue is that of cooperation. If both TF and DnaK both interact throughout the folding trajectory, how do they compete or cooperate? Single-molecule data may show that TF and Hsp70 bind selectively to certain conformations, distinct parts of the same polypeptide, or rather that they both bind the same substrate in a particular temporal order. Questions about temporal order extend to other chaperones, cofactors, and substrate dynamics. For instance, it is unclear how substrate conformational changes are correlated in time with (co)chaperone binding, which may be central to how they guide folding. Another outstanding issue is how chaperones switch between their contrasting cellular roles, for instance between folding guidance and disaggregation. The J-protein co-chaperones, abundantly diversified in eukaryotes, are thought to play a role in modulating the large variety Hsp70 functions [129], though the precise mechanisms are incompletely understood.

How chaperones act at the ribosome is another intriguing issue. For one, chaperone binding may affect the translation rate, as has been suggested for TF in a recent study by

Nilsson et al. [116]. Chaperone binding may depend on the nascent chain folding state, and conversely, chaperone binding may alter substrate folded states. How these interdependencies play out remains unclear. At a more basic level, it is also incompletely understood how many chaperones bind nascent chains during translation, and for how long. Little is known about the interplay between different chaperones at the ribosome in terms of their binding competition or dependencies, with the association of one chaperone for instance being triggered by the action of another.

Addressing these questions on chaperone-substrate dynamics is now getting within reach. Mechanical manipulation of actively translating nascent chains has recently been demonstrated [97]. A number of other approaches are also emerging that will be central to this effort, in addition to existing biochemical and structural assays, such as NMR and deuterium exchange mass spectrometry of protein complexes [64, 87], to ribosomal profiling [81] and single molecule fluorescence techniques [58]. Combining mechanical manipulation with single molecule fluorescence will also open up new possibilities, and may for instance allow one to temporally correlate folding and chaperone binding events. Together, this new array of tools has the potential to provide a mechanistic understanding of how chaperones control protein conformations.

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