Protein folding *in vivo*: the importance of molecular chaperones

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The contribution of the two major cytosolic chaperone systems, Hsp70 and the cylindrical chaperonins, to cellular protein folding has been clarified by a number of recent papers. These studies found that, *in vivo*, a significant fraction of newly synthesized polypeptides transit through these chaperone systems in both prokaryotic and eukaryotic cells. The identification and characterization of the cellular substrates of chaperones will be instrumental in understanding how proteins fold *in vivo*.

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Abbreviations

CCT	chaperonin-containing TCP-1
Hsc	heat-shock cognate protein
Hsp	heat-shock protein
NAC	nascent-chain associated complex
TCP-1	tailless complex polypeptide-1
TF	trigger factor
TRiC	TCP-1 ring complex

Introduction

As a newborn polypeptide emerges into the world, its first contacts with the cellular environment may be critical for determining its fate. Ribosome-bound nascent polypeptides are confronted by a unique set of dangers that must be avoided on the way to achieving a mature, native conformation. Fortunately, a remarkable mechanism involving molecular chaperones has evolved to safeguard the folding of nascent chains. While progress has been made in understanding the basic mechanisms of chaperone action, the contribution of chaperones to *de novo* cellular folding has remained poorly understood and controversial. Although chaperones are clearly important for protein folding and cellular viability, it has been argued that only a few essential proteins require chaperones to fold correctly, whereas the majority of proteins fold spontaneously. An alternative possibility stems from the broad specificity of chaperone binding *in vitro*: as nearly every unfolded polypeptide has the potential to bind chaperones, all newly translated polypeptides might transiently associate with chaperones. A number of new studies have now addressed this problem experimentally and have begun to define the role of chaperones in the folding of newly translated polypeptides. This review summarizes their major findings.

The folding problems of newly translated polypeptides

It is generally accepted that the information necessary to specify the native three-dimensional structure of a protein is inherent in its complete amino acid sequence [1]; however, efficient, reversible folding and unfolding is generally observed only for small proteins. Refolding experiments often lead to the formation of kinetically trapped intermediates that aggregate, even in dilute aqueous solutions and at low temperature [2]. As aggregation is at least partly driven by hydrophobic interactions, it is even more pronounced when folding is attempted under the physiological conditions prevalent in the cell. In particular, the very high concentration of macromolecules creates conditions of crowding that highly favor aggregation (reviewed in [3]).

The folding of newly translated polypeptides faces an additional constraint, as it must be accomplished in the context of the vectorial protein synthesis process. The N-terminal portion of a nascent polypeptide could, in principle, fold spontaneously as it emerges from the ribosome, however, the cooperative nature of the interactions that stabilize folded structures requires that a complete folding domain (50–200 amino acids) be available for productive folding. Furthermore, translation occurs on a timescale of seconds (in bacteria) to several minutes (in eukaryotes), much slower than the millisecond timescale of hydrophobic collapse. Similar dangers exist for proteins during their vectorial import into mitochondria, chloroplasts or the endoplasmic reticulum, into which polypeptide chains are translocated in an extended conformation. Although recent studies indicate that co-translational domain folding simplifies the folding problems encountered by multidomain proteins [4,5,6•,7,8], a growing polypeptide must still be prevented from misfolding and aggregation until a chain length suitable for productive folding has been synthesized. Mounting evidence now indicates that molecular chaperones interact with and stabilize nascent and translocating polypeptides *in vivo* and prevent nonproductive reactions, such as aggregation. Two major classes of ATP-dependent chaperone, the Hsp70s and the chaperonins, have been implicated in *de novo* protein folding in the cytosol of eukaryotic and prokaryotic cells, as well as in organelles of endosymbiotic origin, such as mitochondria and chloroplasts [9,10•,11]. Although substrate binding by both of these chaperone systems is regulated by nucleotide binding and hydrolysis, Hsp70 and the chaperonins are structurally and functionally distinct, and represent radically different principles of chaperone action. The extensive studies on mechanistic aspects of these chaperone systems have recently been summarized in several excellent reviews [9,10•,11].

The contribution of Hsp70s to *de novo* folding

The Hsp70s, in conjunction with co-chaperones of the DnaJ/Hsp40 family, bind and release short linear peptide segments with a net hydrophobic character; such hydrophobic regions are probably present in all unfolded

polypeptides [9,10•,11]. Association with an Hsp70 results in the stabilization of a polypeptide in an extended conformation, thereby preventing its aggregation. For some model substrates, such as firefly luciferase, this is sufficient to promote folding *in vitro*. In many instances, however, the Hsp70-bound substrate must be transferred to a chaperonin complex for productive folding.

A role for Hsp70 proteins in *de novo* folding was originally suggested by several lines of evidence. The observation both that cytoplasmic Hsp70 associated with ribosome-bound nascent chains in eukaryotic cells [4,12–15] and that mitochondrial and endoplasmic reticulum Hsp70s bound to translocating polypeptides [16,17] led to the suggestion that Hsp70s play a general role in stabilizing a translating or translocating polypeptide to prevent its premature misfolding. Supporting this idea, genetic and biochemical studies in *Saccharomyces cerevisiae* demonstrated that the yeast Hsp70 homologs *SSA1–4* are essential for viability [18] and assist the *in vivo* folding of model proteins [19•]. Furthermore, another class of yeast Hsp70, the Ssb proteins, associates stably with ribosomes and can be cross-linked to nascent chains [12,20••].

These experiments did not, however, identify the overall contribution of Hsp70 to *de novo* protein folding *in vivo*. This question was initially addressed using pulse-chase experiments in mammalian cells, whereby the flux of newly translated polypeptides through Hsp70 was assessed by quantitative immunoprecipitation [21••]. These experiments demonstrated that Hsp70 associates transiently with a broad spectrum of polypeptides larger than 20 kDa. Interestingly, a large fraction of these polypeptides are greater than 50 kDa in size. The size of individual domains in cytosolic proteins is approximately 25–30 kDa; hence, the substrates of Hsp70 probably include multidomain proteins that fold co-translationally. In contrast, smaller proteins may have a more limited requirement or weaker affinity for Hsp70. Maximal association with Hsp70 was observed at early chase times and only a small fraction of labeled polypeptide remained associated after 30 min chase. Interestingly, the kinetics of dissociation varied for different substrates, implying that folding of some proteins may require multiple cycles of binding and release. Quantitative analysis indicated that approximately 15–20% of newly synthesized proteins transit through Hsp70 during their biogenesis; however, this is probably an underestimate, as the stringency of the co-immunoprecipitation method does not allow detection of weakly bound or rapidly dissociated substrates.

Early studies of the major bacterial Hsp70, DnaK, did not support a direct role in chaperoning nascent chains. ΔdnaK strains are viable, albeit heat-sensitive, indicating that this chaperone is dispensable for normal growth [22]. Furthermore, their viability does not arise from a functional overlap with another bacterial Hsp70 homolog, HscA, as the doubly deleted strain is also viable [23•]. These findings called into question the proposal that Hsp70s play an essential and evolutionarily conserved role in the folding of newly synthesized proteins; however, a direct role for DnaK in chaperoning bacterial nascent chains has now been established [24••,25••]. Pulse-chase analysis indicated that DnaK interacts transiently with newly synthesized polypeptides over a broad size range, from 14 kDa to well over 90 kDa, binding preferentially to chains ranging from 30 to 75 kDa. Overall, approximately 10% of all soluble polypeptides are associated with DnaK at the earliest chase times and the bulk of these proteins dissociated within 2 min. The association of DnaK with nascent chains was examined by taking advantage of the fact that puromycin-released nascent chains become C-terminally tagged with puromycin and, hence, may be co-immunoprecipitated with antipuromycin antibody. At least 20% of DnaK-bound polypeptides could be reprecipitated using antipuromycin antibody [24••]. This finding confirms the co-translational interaction of Hsp70 with nascent chains in *Escherichia coli* and argues for a general role of Hsp70 in preventing protein misfolding at the ribosome.

If DnaK does indeed associate with nascent chains, why are cells unaffected in its absence? Only one other chaperone component, the trigger factor (TF) protein, is known to bind nascent chains in *E. coli* [26]. The functional significance of this interaction was also unclear, as cells lacking TF (Δtig) are also viable [27]. The absence of TF results in a 2–3-fold increase in the amount of polypeptide associated with DnaK, suggesting that TF and DnaK cooperate in chaperoning nascent chains [24••,25••]. This functional overlap resonates with results from genetic crosses indicating that Δtig and ΔdnaK are synthetically lethal. In the double-mutant strains, both newly synthesized and pre-existing proteins aggregated, with cytosolic proteins appearing to be most susceptible [25••]. These studies indicate that, together, DnaK and TF constitute an essential system for ensuring the productive folding of a substantial fraction of proteins in bacteria. An interesting lesson provided by these studies is that the chaperone system that can functionally replace DnaK *in vivo* is not an Hsp70 homolog, but is an altogether different class of 'small' chaperone. Future studies comparing the substrate binding motifs recognized by both chaperones, as well as their mechanisms of release of bound substrates, may clarify how TF and DnaK can bind to and promote the folding of the same protein subset *in vivo*.

The role of Hsp70 in *de novo* folding appears to be conserved in evolution. However, a comparison of eukaryotic and prokaryotic Hsp70 function reveals that nascent chains in the eukaryotic system remain bound to Hsp70 for longer than in bacteria, with a half-time of dissociation of approximately 10 min. The greater proportion of nascent polypeptides associated with Hsp70, coupled with the decreased dissociation time, implies a more prominent role for Hsp70s in eukaryotic protein folding. Although eukaryotic homologs of TF have not been described, it is, in principle, possible that yet-to-be-identified component(s),

such as the nascent-chain associated complex (NAC) [28], can partially replace or cooperate with Hsp70 in stabilizing nascent chains in eukaryotes. The recently described prefoldin/GimC complex [29•,30•] has been proposed to fulfill a similar function in stabilizing newly translated actin [31•]; however, another study indicates that this complex acts at a later, post-translational stage in the folding pathway and assists chaperonin-mediated folding [32••]. Thus, the exact function of GimC remains a subject for future investigation.

The contribution of chaperonin complexes to *de novo* folding

The chaperonins are large cylindrical protein complexes consisting of two stacked rings of seven to nine subunits each [10•,11]. Group I chaperonins, such as GroEL from *E. coli* and Hsp60 in mitochondria and chloroplasts, function in conjunction with a ring-shaped cofactor, GroES or Hsp10, respectively, which forms the lid on a cage in which polypeptide substrates are enclosed during folding [10•,11]. In contrast, such a cofactor has not been found for the distantly related group II chaperonins from archaea and eukarya. The chaperonin of the eukaryotic cytosol, termed TRiC or CCT (for TCP-1 ring complex or chaperonin-containing TCP-1, respectively, where TCP-1 is tailless complex polypeptide-1), also forms a cage-like structure, but it is hetero-oligomeric, containing eight different subunits per ring (reviewed in [33,34•]). Unlike Hsp70s, chaperonins appear to interact with nonlinear hydrophobic determinants exposed in compact folding intermediates [4,35•,36].

Early studies of Hsp60 function in mitochondria and chloroplasts suggested that chaperonins play an important role in mediating protein folding and assembly. Estimates of the contribution of the bacterial chaperonin GroEL to folding have ranged from barely 2–4% of cellular proteins [37] to approximately 30% [38]. Experiments directly analyzing the flux of newly synthesized proteins through GroEL indicated that it transiently associates with approximately 12% of all newly synthesized proteins; this figure increases 2–3-fold during heat shock [39]. The majority of these substrates range between 10 and 55 kDa and are enriched for a specific subset of approximately 300 polypeptides [40••]. Given the size constraints estimated for the central cavity of GroEL, the upper-size limit observed for physiological substrates is remarkably consistent with polypeptide folding within the cavity. Perhaps most dramatically, overexpression of GroEL increases the fraction of chaperonin-bound polypeptides, but does not change the overall distribution of substrates. This implies that the cellular concentration of GroEL is normally limited to permit only a fraction of available substrates to transit through the chaperonin [39]. Several associated proteins continue to interact with GroEL throughout the course of their lifetime, indicating that, in addition to folding, the chaperonin may also play an important role in the structural maintenance of mature cellular proteins. Interestingly, structural analysis of over 50 natural GroEL substrates revealed a significant preference for proteins composed of multiple α/β domains [40••]. As β sheets are assembled from discontinuous regions of the polypeptide, the binding of these hydrophobic surfaces to GroEL might facilitate the correct packing of strands within the β sheet, as well as the packing of α helices against neighboring β sheets.

The role of the yeast mitochondrial chaperonin system in protein folding was also recently examined, using temperature-sensitive alleles of both Hsp60 and the co-chaperonin Hsp10 [41•]. As previously observed for GroEL, loss of Hsp60 results in a pronounced increase in the aggregation of a wide range of mitochondrial components. Interestingly, the subsets of proteins aggregated in Hsp10 and Hsp60 mutants were not identical, suggesting that some polypeptides may only require the assistance of Hsp60 for folding.

Despite its similarity to bacterial chaperonins, the substrate spectrum of the eukaryotic cytosolic chaperonin TRiC/CCT has been a matter of controversy. Primarily on the basis of the analysis of TRiC/CCT mutants in *S. cerevisiae*, which exhibit cytoskeletal defects characteristic of defective actin and tubulin function, it has been suggested that TRiC is a specialized chaperone that folds only a few cytoskeletal proteins [42]. In contrast, direct examination of the substrate spectrum of TRiC/CCT using pulse-chase analysis in mammalian cells demonstrated that 9–15% of newly synthesized proteins transit through the chaperonin [21••]. As observed for Hsp70 and GroEL, the dissociation kinetics from TRiC varied for different proteins, suggesting a differential requirement for cycles of binding and release. Interestingly, most TRiC-bound proteins were between 30 and 60 kDa in size. The restricted size range observed for cellular TRiC substrates bears parallels to the studies of GroEL substrates and lends further support to the idea that chaperonin-mediated folding occurs within an enclosed central cavity [33,34•]. Nonetheless, several large proteins of 100–120 kDa also transit through the chaperonin, raising the possibility of domain-wise folding of larger proteins by TRiC. Analysis of TRiC-associated substrates on two-dimensional gels identified at least 70 distinct substrate polypeptides. The identity and structural features that characterize cellular TRiC substrates remain to be defined; however, studies using model proteins have expanded the list of known TRiC substrates to include, in addition to actin and tubulin-related proteins, luciferase [4], G alpha transducin [43], cyclin E [44] and myosin [45]. On the basis of the structure of these known examples, TRiC substrates may have a complex domain organization that results in folding intermediates with a higher tendency to aggregate; alternatively, they may share a requirement for binding to either a cofactor or an oligomeric partner in order to complete folding. Given that most of the heterogeneity among TRiC subunits resides in the putative substrate-binding domain [33,34•], it is possible that different subunits in the complex have evolved to recognize different motifs in substrate proteins.

Networks, pathways and the organization of chaperone action in the cell

Recent years have witnessed a spirited debate concerning the extent of functional integration among the various chaperone systems in the cell [46,47]. Two models have been proposed to describe how chaperones mediate *de novo* folding [43,48–50]. According to one, the folding of newly synthesized proteins is a highly coordinated process involving the sequential and processive interaction of different chaperone systems with folding intermediates [49,50]. The alternative model holds that chaperones interact with substrate proteins in a stochastic manner and that non-native folding intermediates partition freely through the cytosol, cycling between a network of available chaperones and the machinery for proteolytic degradation [43,51]. Because a small fraction of the polypeptides reach the native state in each cycle, a major difference between the models is that, according to the partitioning model, non-native folding intermediates are fully discharged into the bulk cytosol multiple times before reaching the native state [43]. In contrast, the processive model proposes that the newly translated polypeptide is released into the bulk cytosol once it has adopted a conformation that is committed to fold. To discriminate between these models, the processivity of *de novo* folding was examined in both yeast and mammalian cells by introducing a GroEL mutant (D87K GroEL) that acts as a trap for non-native folding intermediates [21••,32••]. D87K GroEL binds promiscuously to non-native proteins and is unable to release them (reviewed in [10•,11]). Indeed, when expressed in the cytosol of yeast or mammalian cells, D87K GroEL was fully capable of binding stress-denatured proteins, as well as newly translated polypeptides that were unable to fold. However, the D87K GroEL trap was unable to bind to the folding intermediates generated during protein synthesis, which associated instead with the endogenous cytoplasmic chaperones. These experiments support the view that folding *in vivo* is mediated by a highly organized chaperone machinery that is functionally coupled to translation. They also suggest that the mechanisms that determine the fate of misfolded or stress-denatured proteins involve the cycling of non-native forms between cellular components and the cytosol, as proposed by the partitioning model.

At a mechanistic level, the coupling of folding and translation (or translocation) might be accomplished by the specific recruitment of chaperone components to either the translation machinery or the translocation machinery. For instance, TF is directly associated with bacterial ribosomes [26]. Hsp70 binding to substrates appears to be governed by association with proteins carrying the characteristic 'J-domain', which functions as a recruitment site for Hsp70 [52]. DnaJ family chaperone proteins contain additional domains that serve as localization signals, which target the various DnaJ homologs to a particular subcellular location or organelle. These include TIM44, a component of the mitochondrial import machinery [53], and Sec63, a component of the endoplasmic reticulum translocon [17]. In the eukaryotic cytosol, potential candidates for recruiting Hsp70 to bind nascent chains include Hsp40 [4], the J-domain protein zuotin, which also contains a charged region essential for ribosome association [54•], and NAC [28]. Yet another recruitment mechanism appears to be functional in chloroplasts, in which IAP100, a component of the translocation machinery, directly recruits Hsp60 [55].

The sequential nature of chaperone interactions *in vivo* was originally suggested by experiments that examined the folding of model proteins either imported into mitochondria or chloroplasts [16,56,57], or translated in cell-free extracts [4], as well as by experiments using purified chaperone components [58,59]. In these systems, the polypeptide was initially bound and stabilized by Hsp70, and subsequently transferred to a chaperonin. The recent examination of chaperone–substrate interactions *in vivo* is consistent with the sequential interaction model [24••]. Analysis of the transit of newly made polypeptides through bacterial chaperones indicated that overexpression of GroEL increases the flux of substrates through DnaK, as expected if the chaperonin is downstream in the folding pathway. Notably, TF, which functionally replaces DnaK in ΔdnaK strains, also appears to cooperate with GroEL in substrate binding [60]. It is thus possible that the cell has evolved redundant pathways of polypeptide transfer from 'small chaperones' (i.e. Hsp70 and TF) to chaperonins. It is not clear how substrate polypeptides are transferred among chaperone systems. It is possible that different conformations of the substrate occur along the folding pathway and are specifically recognized by different chaperones; however, it is also possible that adaptor proteins or direct interactions among the chaperones themselves bridge the transfer reaction.

An emerging model for chaperone action *in vivo*

Through these studies, a more coherent picture of how proteins fold *in vivo* is now beginning to emerge. Despite important differences between prokaryotic and eukaryotic protein folding, such as their ability to promote co-translational folding [5], there are also striking parallels between the two kingdoms (Figure 1). Quantitative analysis of chaperone interactions revealed that a large fraction of newly translated proteins flux through the major chaperone systems in the cell. Newly translated polypeptides interact first with so-called 'small chaperones', including Hsp70 and TF (Figure 1a). The ability of these chaperones to prevent aggregation is probably sufficient to promote the folding of a large subset of polypeptides; however, a considerable number of polypeptides also require the protected folding environment provided by the central cavity of prokaryotic and eukaryotic chaperonin complexes (Figure 1b). Perhaps these proteins have a more complex, aggregation-prone domain structure that requires extensive interactions among noncontiguous regions. Most chaperonin substrates are medium-sized proteins, between 25 and 60 kDa. This observed size distribution suggests

Figure 1

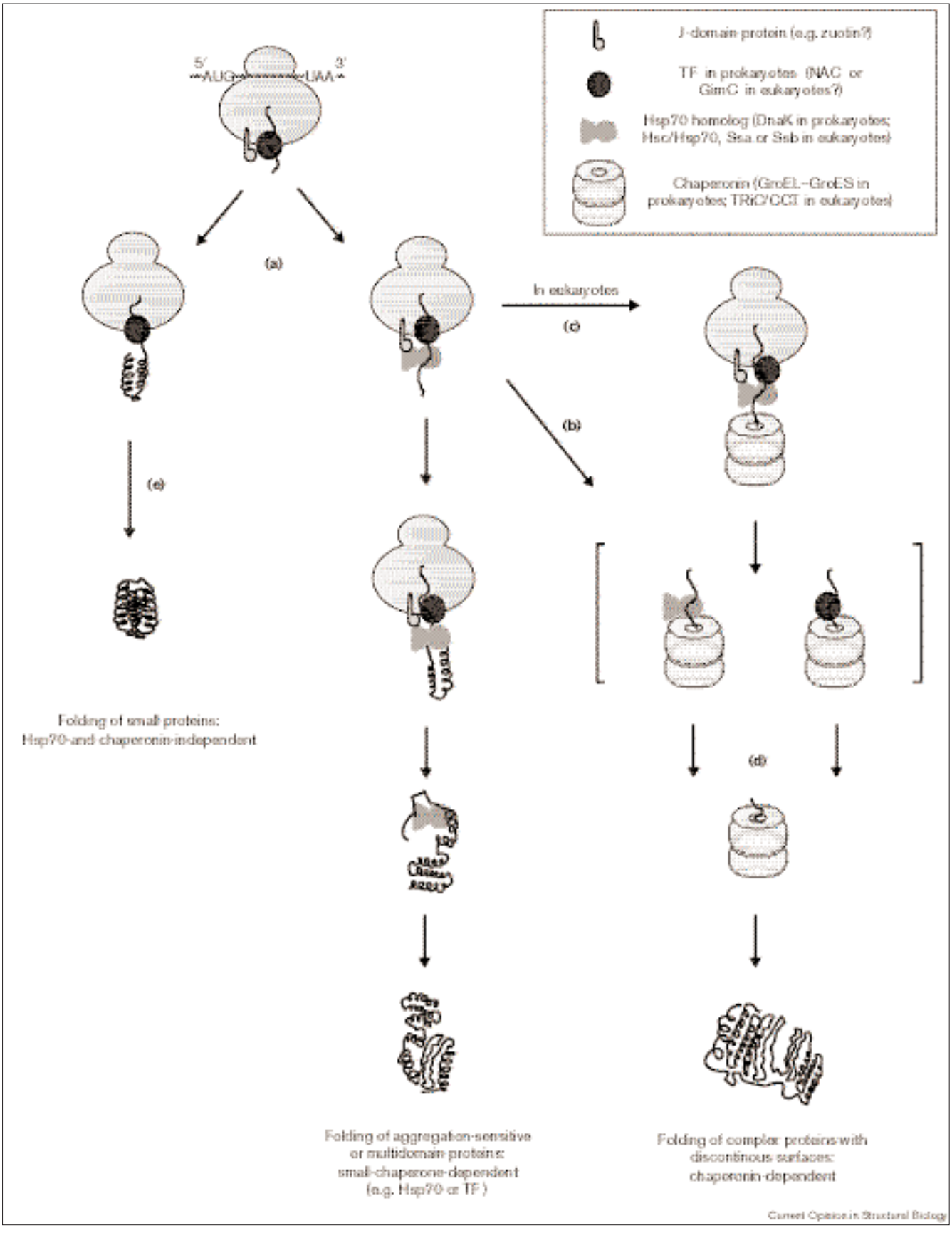
J-domain protein (e.g. zuotin?)
TF in prokaryotes (NAC or GimC in eukaryotes?)
Hsp70 homolog (DnaK in prokaryotes; Hsc/Hsp70, Ssa or Ssb in eukaryotes)
Chaperonin (GroEL–GroES in prokaryotes; TRiC/CCT in eukaryotes)
5′
AUG
UAA
3′
(a)
In eukaryotes
(c)
(b)
(e)
(d)
Folding of small proteins:
Hsp70- and chaperonin-independent
Folding of aggregation-sensitive or multidomain proteins:
small-chaperone-dependent
(e.g. Hsp70 or TF)
Folding of complex proteins with discontinous surfaces:
chaperonin-dependent
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Figure 1 legend

Schematic representation of *de novo* protein folding in the cytosol of prokaryotic and eukaryotic cells. The model emphasizes the evolutionarily conserved characteristics of the folding process; however, some aspects are specific to either prokaryotic or eukaryotic cells. For instance, co-translational domain folding, as well as association of the chaperonin complex with nascent chains, is favored in eukaryotes. Conversely, no homolog of TF has been identified in eukaryotes (although several candidates exist). For simplicity, cofactors of Hsp70 and chaperonin, and alternative folding pathways involving other chaperones (e.g. Hsp90) are not represented. See text for details.

that very small proteins do not need the protected environment of the chaperonin cavity to fold. Conversely, large proteins too large to fit are probably composed of smaller individual domains that can fold co-translationally. The lack of a GroES-like cofactor in eukaryotes might allow the co-translational binding of one domain to the chaperonin (Figure 1c); this might be the case for firefly luciferase, whose N-terminal domain folds co-translationally [4], and for myosin, whose N-terminal motor domain also associates co-translationally with the chaperonin [45]. Although the sequential interaction of newly synthesized polypeptides with small and large chaperones has been observed in both prokaryotes and eukaryotes, and is possibly mediated by direct interactions (Figure 1d), it is possible that some proteins bind directly to the chaperonins.

An interesting corollary of these studies is that a substantial fraction of cellular proteins appear to fold without the assistance of either Hsp70 or the chaperonins (Figure 1e). How do these proteins reach the native state? The folding of specific subsets of cytosolic proteins may occur in an unassisted manner or may be carried out by novel, uncharacterized chaperone systems. For example, Hsp90 does not appear to play a general role in *de novo* folding [61], but is required for folding a restricted class of proteins that includes steroid hormone receptors and Src-like tyrosine kinases (reviewed in [62]). Interestingly, these substrates are also reported to require a sequential interaction with Hsp70 prior to transfer to Hsp90 (reviewed in [63]). In addition, the translational machinery itself may also possess some chaperone-like functions, such as prevention of aggregation [64,65].

What determines whether the folding of a certain protein requires chaperone assistance? The *in vivo* analysis of protein folding indicates that intermediates with exposed hydrophobic surfaces are not released into the bulk cytosol, except under stress conditions. Thus, it is probable that if a newly translated polypeptide exposes hydrophobic surfaces it will be targeted to the chaperone machinery. In contrast, small proteins with rapid folding kinetics, as well as proteins consisting of small domains that form co-translationally, may not engage in stable or detectable interactions with cytosolic chaperones.

Perspectives and future directions

Studies defining the role of the Hsp70s and chaperonins in the folding of a large fraction of cellular proteins raise critical questions stemming from the discrepancy between the substrate repertoire observed *in vivo* and *in vitro*. *In vitro*, both GroEL and Hsp70 interact promiscuously with most unfolded proteins. The observation that only a discrete fraction of the large constellation of cellular polypeptides actually interact *in vivo* with either of these chaperones raises the question of how this specificity is achieved. Importantly, this may be determined, in part, by the conformation adopted by nascent polypeptides emerging from the ribosome. Thus, an important area of research will be to understand how co-translational folding events influence the folding pathway of proteins.

The identification and characterization of *in vivo* chaperone substrates may be a prerequisite for a better understanding of folding processes in the cell. This will be a challenging task and will probably require the use of global proteomics approaches. In the answer, however, may lie the fundamental rules of protein folding in the cell, with their staggering implications for our understanding of protein regulation under normal conditions and in the generation of disease.

Note added in proof

Four recent publications [66••,67••,68•,69••] represent important advances in our understanding of chaperone function.

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