Protein folding: **Who chaperones nascent chains in bacteria?**

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The physiological roles of the molecular chaperones trigger factor and DnaK in *de novo* protein folding have been unclear, but two new studies have shown that they perform essential, yet partially redundant, functions in chaperoning nascent protein chains in bacteria.

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Important advances in biology often occur when problems that have been controversial and seemingly complex are resolved with surprisingly simple and clear answers. Such has been the case with the problem of how proteins fold inside cells, which has seen a number of controversies and solutions in the past decade. A very recent example of this concerns the physiological functions of two molecular chaperones, trigger factor and DnaK, in protein folding in the cytosol of a bacterial cell. The *in vivo* roles of these two molecular chaperones have been questionable, as bacteria lacking either of them are viable. But recent studies have shown that they cooperate to chaperone nascent protein chains as they fold, and that together they are indeed essential for cell viability.

Protein folding and the chaperone concept

I shall start with some background information on the basic principles of protein folding and chaperone function. The amino-acid sequence of a protein is the crucial determinant of its native three-dimensional structure. Many proteins can fold spontaneously in the test tube as long as they do not undergo aggregation or interact inappropriately with themselves or other proteins. In the living cell, however, the protein concentration is extremely high, so that there are many chances for newly synthesized proteins to make incorrect interactions. Furthermore, a nascent polypeptide chain may not be able to fold completely while it is being made on a ribosome, as sequences required to attain the native conformation may be buried in the ribosomes or not yet synthesized. And as the amino-terminal end of a nascent chain is extruded from the ribosome, hydrophobic residues that are buried in the final structure may be exposed, capable of associating with other polypeptides in a non-productive manner. It was therefore reasonable to assume that the cell has specific mechanisms for preventing the misfolding and aggregation of newly synthesized proteins. An important step towards a molecular understanding of how proteins fold inside cells came with the discovery of molecular chaperones [1–3].

Molecular chaperones are protein machines that recognize non-native states of other proteins and, by controlled binding and release, assist these substrate proteins to fold properly. The most familiar molecular chaperones were originally identified as ‘heat-shock’ or ‘stress’ proteins — proteins that are induced under conditions likely to generate increased levels of aggregated or denatured proteins. It soon became evident, however, that the various families of heat-shock proteins (Hsps) are also involved in numerous cellular processes under normal growth conditions [1–3]: the folding of newly synthesized proteins; the transport of precursor proteins to and across biological membranes; the modulation of the oligomeric state of protein complexes; and the facilitation of protein degradation.

A pathway for protein folding

The two best-studied families of chaperones are the Hsp70s and the Hsp60s (or ‘chaperonins’). Representatives of the Hsp70 family have been found in prokaryotes and most cellular compartments of eukaryotes [1–3]. They have two primary domains: an ATPase domain and a peptide-binding domain. They bind via the latter domain to segments of unfolded polypeptides, particularly those containing hydrophobic residues, and release them in an ATP-dependent manner. Co-chaperones have been identified that cooperate with Hsp70s: one is Hsp40, or DnaJ, which increases the rate of ATP turnover by Hsp70s and can itself directly interact with substrate polypeptides; another is GrpE, which has been found in bacteria and mitochondria and facilitates nucleotide release from Hsp70.

The Hsp60s have been identified in bacteria — where the representative is the well-studied GroEL — mitochondria and chloroplasts [1–3]. They assemble into large, double-ring structures and, together with the co-chaperonin known as Hsp10 or GroES, provide a central cavity that allows proteins of size up to about 60–65 kDa to fold in a protected environment [4]. The GroEL–GroES reaction cycle also involves the hydrolysis of ATP. A second type of chaperonin has been found in archaebacteria and the eukaryotic cytosol, and is known as the ‘chaperonin containing t-complex polypeptide’ (CCT) or ‘TCP-1 ring complex’ (TRiC) [1,5]. These also form double-ring structures, but they do not appear to have a GroES-like co-chaperonin and apparently interact with only a limited set of substrate proteins.

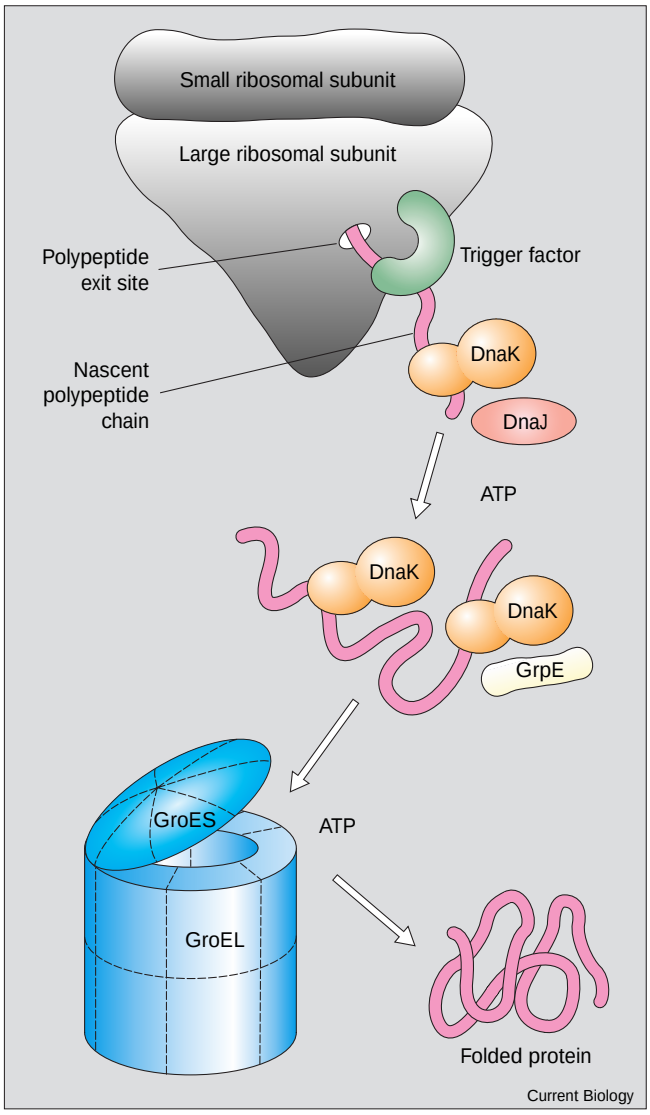
Studies of protein folding in mitochondria [6–9], followed by detailed analysis of protein folding *in vitro* with purified bacterial chaperones [10], have led to the following proposed folding pathway for a polypeptide chain. An unfolded polypeptide chain first interacts with the Hsp70 system. The major bacterial Hsp70, DnaK, in cooperation with DnaJ, binds to exposed hydrophobic segments of the nascent polypeptide chain and prevents misfolding or aggregation. DnaJ and the nucleotide-release factor GrpE promote the ATP-driven reaction cycle of DnaK. Upon its release from DnaK, the polypeptide chain is transferred onto the chaperonin system — GroEL–GroES in a bacterial cell — where the protein can fold in the central cavity, again with the expense of ATP [1,3]. Depending on the protein substrate, a single round or multiple rounds of interaction with these chaperones are required to complete the folding process.

In the case of the eukaryotic cytosol, clear evidence has accumulated that Hsp70s interact with a wide range of polypeptide chains during their synthesis on ribosomes [11,12]. Neither GrpE nor a GroEL-type chaperonin has been found in the eukaryotic cytosol, yet additional chaperones and co-chaperones have been shown to be involved in protein biogenesis in this compartment [1,2]. The heterodimeric complex known as the 'nascent chain associated complex' (NAC) probably interacts first with the nascent polypeptide chain as it emerges at the exit site of the ribosome [13]. The chaperonin TRiC can interact with nascent chains of a subset of proteins, apparently after NAC and Hsp70 have associated with the chain [1].

While the exact roles of NAC and TRiC remain to be established, it is generally accepted that Hsp70 systems play a major role in *de novo* protein folding in all the major compartments of a eukaryotic cell [11,12,14,15]. Even for proteins that are translocated into the lumen of the endoplasmic reticulum in a co-translational manner, the association of the nascent polypeptide chains with a lumenal Hsp70 — known as BiP or Kar2 — is important for their proper folding [16]. In the case of mitochondria, unfolded pre-proteins are generally transported post-translationally across both membranes into the matrix, where they interact with an Hsp70 that facilitates both their translocation and folding [8,9,17].

In the bacterial cytosol, GroEL interacts with newly synthesized polypeptides post-translationally — that is, after release from the ribosome — and neither NAC nor TRiC are present [1,3]. Until recently, evidence that the DnaK (Hsp70) system plays an important role in the *de novo* folding of nascent polypeptides in bacteria has been elusive (see below), and this was taken as an indication that there might be fundamental differences between the protein folding machines of bacteria and eukaryotes.

Figure 1

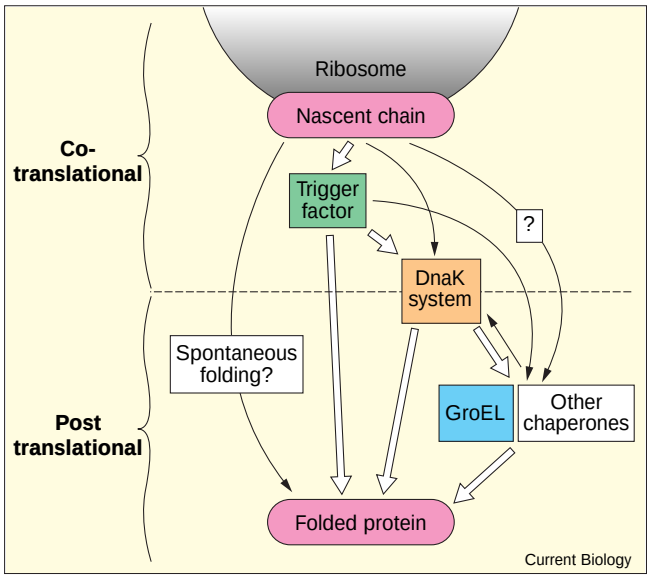


Folding pathway for a model protein involving three chaperone systems in the bacterial cytosol. (See text for details.)

Trigger factor – a ribosome-associated chaperone

In recent years, a bacteria-specific ribosome-associated protein has become an attractive candidate as a chaperone for nascent polypeptides. This is the 'trigger factor', a 48 kDa protein which was first identified as a putative molecular chaperone for secretory proteins in *Escherichia coli* [18]. In an independent study [19], a search for prolyl isomerases — enzymes which catalyze the isomerization of the peptide bond preceding a prolyl residue that is necessary for proper folding of some proteins — led to the discovery of a ribosome-associated enzyme that was identified as trigger factor. Trigger factor was subsequently rediscovered as a factor that interacts directly with nascent polypeptide chains at *E. coli* ribosomes [20,21].

Figure 2



Folding pathways for different proteins in the bacterial cytosol. (See text for details.)

Trigger factor has three domains: an amino-terminal ribosome-binding domain, a middle domain with prolyl isomerase activity, and a carboxy-terminal domain for which no function has been clearly defined [22,23]. In contrast to other prolyl isomerases, trigger factor binds tightly to unfolded polypeptides, an ability that is important for its chaperone-like functions [23]. The middle domain alone has full prolyl isomerase activity towards short peptides, but a strongly reduced ability to facilitate protein folding. Trigger factor seems to have independent sites for catalysis and binding unfolded proteins; the latter (chaperone-like) site even recognizes protein substrates lacking prolyl residues [23]. The chaperone function has not been clearly localized within trigger factor, though it has been shown to require the carboxy-terminal domain and additional parts of the protein. It is not known which function of trigger factor plays the dominant part in *de novo* protein folding, although some preference may be given to the chaperone function at normal temperatures.

Do bacterial nascent chains require a chaperone *in vivo*?

The *in vitro* results are compatible with a folding pathway for newly synthesized bacterial proteins in which trigger factor is the initial chaperone, followed by the DnaK system and GroEL/GroES — an extension of the folding pathway that was originally proposed and is described above (Figure 1). In support of this, the three chaperone systems described here are typically conserved in evolution [1] — even bacteria with very small genomes appear to have trigger factor, the DnaK system and GroEL/GroES. But only the GroEL/GroES system appears to be essential for cell viability under all growth conditions [24].

The analysis of *E. coli* cells lacking either trigger factor or DnaK raised serious doubts about the requirement for chaperones for *de novo* protein folding on ribosomes under normal growth conditions. At normal temperatures, mutant cells were found to live well without trigger factor, indicating that trigger factor's chaperone and prolyl isomerase activities are both dispensible for protein folding *in vivo* [25,26]. At very low temperatures, trigger factor was required for cell survival [27]; under these conditions, its prolyl isomerase activity becomes crucial, probably because the uncatalyzed isomerization of peptidyl–prolyl bonds is very slow at these temperatures [26]. Even the deletion of DnaK (and DnaJ) is tolerated by bacterial cells at normal temperatures: DnaK-deficient cells lacked obvious defects in *de novo* protein folding [28]. A double mutant cell lacking both DnaK and HscA — an additional, low-abundance Hsp70 of *E. coli* — also failed to show any protein folding defect at intermediate temperatures [28].

It was concluded from these observations that bacterial Hsp70 is not required to chaperone nascent polypeptide chains. DnaK-deficient cells did, however, show defects in the repair of misfolded proteins that accumulated under stress conditions. The major function of DnaK thus appeared to be in post-translational processes, particularly the refolding of proteins under stress conditions [28]. These findings raised the question of whether nascent polypeptide chains in the bacterial cytosol require chaperones at all, or whether they can fold spontaneously. The answer to this question has recently been provided by two studies [25,26] that have elegantly reconciled the different views.

Cooperation of trigger factor and DnaK

In these new studies [25,26], numerous newly-synthesized proteins, including nascent chains, were found by co-immunoprecipitation to be associated with DnaK in wild-type *E. coli* cells. In cells lacking trigger factor, about two–three-fold more proteins were found associated with DnaK, indicating that DnaK substitutes in part for the function of trigger factor [25,26]. When the levels of DnaK and DnaJ were reduced in the trigger-factor-deficient cells, folding defects were observed for a large number of cytosolic proteins [25]. The absence of both trigger factor and DnaK was found to be lethal — the cells were inviable, demonstrating that the two chaperones functionally cooperate [25,26]. The viability of cells lacking just one or other chaperone can thus be explained by their partial functional redundancy. Their overlapping function in *de novo* protein folding is apparently so important that two quite different chaperone systems evolved to perform this task.

Protein flux through bacterial chaperone systems

The model illustrated in Figure 1 holds true for a number of bacterial proteins [26], but the three chaperone systems are not all required for folding of every protein. The new results suggest that, for many proteins, only one or two of the chaperone systems are involved (Figure 2). In wild-type cells, about 10–15 % of newly-synthesized proteins were found to interact with DnaK [25,26] and a fraction of similar size was observed to be associated with GroEL [29,30]. In trigger-factor-deficient cells, 20–35 % of the newly-synthesized proteins were associated with DnaK, whereas only a slight increase was observed in the proportion associated with GroEL [25,26]. These values are probably underestimates, as the complexes may partially dissociate during co-immunoprecipitation. The fraction of newly synthesized proteins that interact with trigger factor has not yet been determined.

The three chaperone systems show different size preferences. While most substrates of GroEL are smaller than 60–65 kDa, being restricted by the space provided by its central cavity [1,3,4], DnaK preferentially interacts with polypeptide chains larger than 30 kDa and is most likely the key chaperone for proteins larger than 65 kDa [25,26]. Binding of trigger factor to the nascent polypeptide chain at the ribosome exit site may prevent DnaK from interacting with very short nascent chains. Indeed, the proportion of short polypeptides — below 30 kDa — associating with DnaK was significantly increased in trigger-factor-deficient cells [26].

Many important questions about how these chaperone systems work remain to be answered, and I shall mention only a few. Which of the various activities that have been ascribed to trigger factor — ribosome binding, prolyl isomerase and chaperone activity — are crucial for protein folding? The observed cooperation of trigger factor and DnaK in bacteria may prompt a detailed analysis of the functional relation between NAC, ribosome-associated Hsp70s and further factors in eukaryotes. It is also an open question whether protein folding pathways are mainly unidirectional — that is, whether polypeptides sequentially use the various distinct chaperone systems — or whether the chaperone systems function as network, with substrate proteins shuttling back and forth between different chaperones [1–3]. We do not yet know for sure whether some proteins can reach their native state *in vivo* independently of any chaperone (Figure 2), or whether every newly synthesized protein has to interact with at least one chaperone system: a number of small proteins have been shown to fold extremely rapidly *in vitro* [31], and if any proteins undergo spontaneous folding *in vivo* it is likely to be these.

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