Chaperones: Helpers Along the Pathways to Protein Folding

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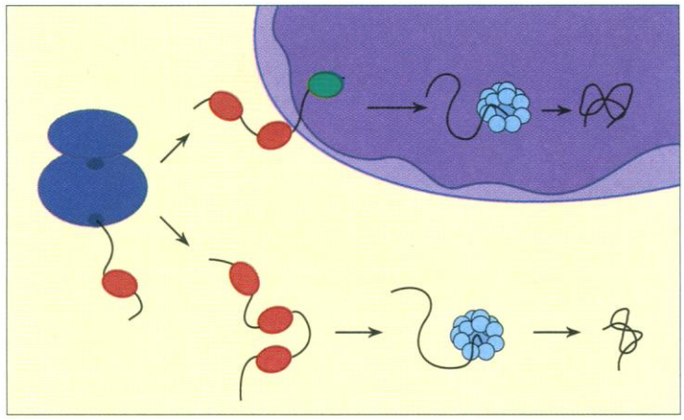
As first exposed by Anfinsen and colleagues 30 years ago (*1*), proteins contain within their amino acid sequences the information necessary for proper folding. Many purified, denatured proteins spontaneously fold into their native conformation. However, the recent identification of a class of proteins that interact with a wide array of polypeptides from the time of synthesis until the final folded state implicates them in the process of protein folding in vivo. These proteins, popularly referred to as molecular chaperones, were studied for many years in another context as heat shock proteins (Hsps), universally synthesized by cells in response to stresses such as increases in temperature (*2*).

Two families of Hsps are most intimately tied to general protein folding in cells: the Hsp70 and the Hsp60 (or chaperonin 60) families. The name of the complex Hsp70 family is derived from the fact that its first member was identified as a 70-kilodalton protein synthesized only when a stress impinged upon a cell. However, most members of this family are present in cells under optimal, nonstressful conditions. In fact, many Hsps are essential for life. Because chaperones were first identified as Hsps, their involvement with proteins partially denatured by stresses has been assumed for sometime. The most plausible roles of Hsps in the stressed cell are the rescue of unfolded or aggregated polypeptides back to an active conformation and the acceleration of the proteolysis of proteins denatured beyond repair. However, because the production of Hsps is induced by stresses that partially denature proteins, it has been unclear whether chaperones are involved directly in protein folding under normal conditions. The sequential interaction of Hsp70 and then Hsp60 with polypeptides in the nonstressed cell, from the time of their synthesis to their final stages of folding, supports the argument for a direct role for Hsps in protein folding (see figure).

This intimate association between Hsps and normal protein synthesis is illustrated by the interaction of cytoplasmic Hsp70s with the nascent polypeptide chain while it is still on the ribosome (*3*). In addition, proteins destined for the endoplasmic reticulum (ER) or mitochondria are bound to cytoplasmic Hsp70s in the cytosol and immediately bind organellar Hsp70s upon emergence into the lumen of the ER or matrix of the mitochondria (*4–6*). In mitochondria, the Hsp70-bound protein is then "passed off" to Hsp60 (see figure). Genetic experiments in yeast demonstrate that Hsp60 is required for proper folding and assembly of proteins into multimeric complexes (*7*).



Hsp70 and Hsp60 in protein folding. Cytosolic Hsp70 (red) binds to nascent chains on ribosomes. **(Top)** Proteins imported into mitochondria bind to mitochondrial Hsp70 (green) upon entrance into the matrix and are then passed to Hsp60 (blue) where folding takes place. **(Bottom)** Hsp60 (blue) may help fold cytosolic proteins (*17*).

The idea of a pathway of chaperone action was extended by Hartl (*8*) with an in vitro system consisting of the *Escherichia coli* Hsp70 (DnaK), Hsp60 (GroEL), and the additional, unrelated Hsps GrpE, DnaJ, and GroES. The temporal interaction of Hsps in this system closely mimics the pathway delineated for the interaction of Hsps in the import and folding of mitochondrial proteins in vivo. In vitro, Hsp70, DnaJ, and Hsp60 interact sequentially with folding polypeptides. The Hsp70 binds to a completely unfolded polypeptide such as rhodanese that does not normally fold properly in vitro. Spectral analysis indicated that rhodanese bound to Hsp70 and DnaJ attains a conformational state intermediate between the unfolded and completely folded tertiary state of the active enzyme. Unfolded rhodanese aggregates upon attaining a partially folded state. However, in the presence of ATP and GrpE, the partially folded rhodanese bound to Hsp70 and DnaJ is efficiently transferred to Hsp60, where folding is completed. These experiments also reveal that Hsps other than Hsp70 and Hsp60 are critical for the efficient function of this system. Homologs of DnaJ and GroES have been identified in eukaryotes, suggesting conservation of this complex chaperone machinery through evolution (*9, 10*).

Chaperones clearly participate in the normal folding of proteins. A much more difficult question is whether the chaperones increase the rate of a limiting, on-pathway folding reaction or decrease the rate of off-pathway reactions, including those leading to aggregation. As the first chaperone to interact with the nascent polypeptide, Hsp70s are likely to prevent nonproductive (that is, off-pathway) interactions, keeping the polypeptide unfolded until productive interactions can occur. This Hsp70 interaction may be particularly important for proteins that require the carboxyl terminus, the last part of the protein to be synthesized, for the folding of the amino-terminal region into its final conformation. The Hsp70 in organelles interacts with the amino termini of the translocating protein before the movement of the remainder of the protein into the matrix. This Hsp70 interaction in mitochondria may play an analogous role to that in the cytoplasm by keeping the protein unfolded until translocation is completed.

On the basis of data obtained in vivo and in vitro, Hsp60 would be the best candidate for a chaperone that actively promotes on-pathway reactions. This family of proteins can facilitate the in vitro folding of proteins such as rhodanese for which conditions for spontaneous folding have not been found. However, an Hsp60-dependent increase has not been shown in the rate of folding of proteins able to fold spontaneously in vitro. For example, the folding of dihydrofolate reductase in vitro is slower than spontaneous folding, probably because of the interactions of the unfolded or partially folded proteins with the chaperones. It is clear, however, that there is a basic difference among the interactions of polypeptides with these chaperones, which is consistent with the sequential interaction of a folding polypeptide with Hsp70 and then Hsp60. One particular peptide binds Hsp70 in an extended conformation, as expected from a nascent chain or polypeptide translocating across an organellar membrane, but binds Hsp60 in a helical state characteristic of an intermediate in folding (*11*).

The Hsp70 family has important roles in the cell beyond protein folding. Although binding of Hsp70 in the lumen of the ER or

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the matrix of the mitochondrium may keep the translocating polypeptide in an unfolded conformation, interaction with Hsp70 in organelles is also essential for the translocation process (*12–14*). After insertion of the amino-terminal leader sequence across the mitochondrial membranes, the vectorial movement of the bulk of a polypeptide into the matrix requires the binding of mitochondrial Hsp70. A simple model of Hsp70 action in translocation shows that its binding provides a directionality to the translocation process by sterically preventing movement of the polypeptide back toward the cytoplasm. Continued movement into the matrix might be accomplished simply by Brownian motion, with backward movement prevented by the binding of Hsp70 to internal sites on the translocating polypeptide. Such a model would require that mitochondrial Hsp70 have a higher affinity for the translocating polypeptide than that of any cytosolic protein, such as cytosolic Hsp70. However, Hsp70s are thought to bind with particularly high affinity to hydrophobic patches exposed in unfolded polypeptides. Because the polypeptide crosses the membranes in an unfolded state (*15*), such hydrophobic sequences could easily bind to mitochondrial Hsp70.

The binding of Hsp70 to the nascent polypeptide chain can also have important consequences on the earliest events in protein maturation, namely translation (*16*). Yeast strains lacking a class of cytoplasmic Hsp70s that associate with translating ribosomes show defects in protein synthesis, including sensitivity to certain antibiotics that inhibit translation. The growth defect of such strains can be overcome by overproduction of a protein related to the translation elongation factor EF1a, the protein responsible for bringing the aminoacyl tRNA to the ribosome. The binding of Hsp70 to the nascent polypeptide chain may be important for the polypeptide's movement out of the tunnel of the 60S ribosomal subunit, in a way analogous to the binding of mitochondrial Hsp70 in translocation of a polypeptide through the membrane into the matrix. The lack of Hsp70 could result in the nascent chain backing up in the tunnel of the 60S subunit, thus distorting the interactions of peptidyl and aminoacyl tRNAs. The critical importance of chaperones in protein synthesis, translocation across membranes, and folding likely has ensured that the primary sequences of proteins have evolved to contain chaperone binding sites in addition to sequence information for attaining the proper tertiary conformation. Because chaperones can bind a variety of peptide sequences, the need for chaperone binding sites would not have unduly constrained the evolution of protein sequences.

The general outline of the interaction of chaperones with newly synthesized proteins is known. However, many questions remain as to the mechanism of action of these ubiquitous proteins and to the precise nature of their physiological roles in vivo. The answers will be unveiled by a combination of genetic analyses to uncover other components involved in these processes, coupled in vitro translation and folding systems to mimic the in vivo situation, and more sophisticated biochemical analyses of the chaperone-mediated protein folding.

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To Fold or Not to Fold ...

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Molecular chaperones are cellular factors that shepherd newly synthesized proteins along the hazardous journey to the folded state. Their discovery has taken the study of protein folding from the arena of the biophysicist into the cell biological limelight. The results of both in vivo and in vitro studies suggest that these molecular chaperones are crucial for the folding and assembly of many multi-domain and multi-subunit proteins. In fact, it is through a combination of biophysical and cell biological approaches that the mechanism of action of the chaperones is now becoming clear.

Protein folding, especially in the extremely concentrated environment of the cell, must be thought of as a kinetic competition between on-pathway reactions leading to the folded state and off-pathway reactions leading to aggregation: $A \rightleftharpoons U \rightleftharpoons F$, where U is the unfolded protein, F is the folded protein, and A represents either reversible or irreversible aggregation. During the course of in vitro refolding reactions, transient states are formed that expose a substantial amount of hydrophobic surface. When such partially folded states are present at moderate concentrations, intermolecular aggregation can occur by way of these hydrophobic patches.

What strategies might the cell use to prevent aggregation? It could (i) block the exposed hydrophobic patches; (ii) create an isolated environment in which each protein molecule can fold independently; or (iii) sufficiently accelerate the on-pathway folding rate to kinetically out-compete aggregation. In fact, nature has exploited each of these approaches. The blockade of exposed hydrophobic patches requires a factor that can reversibly bind to them. The molecular chaperone Hsp70 and its relatives [Hsc70, DnaK, BiP, Kar2p, and Ssa1–4p (*1*)] meet this requirement and are ubiquitous monomeric proteins found in the cytosol, endoplasmic reticulum, and mitochondria. The Hsp70 class of chaperones binds to unfolded and partially folded states of a variety of proteins but shows little interaction with native, folded proteins (*2*). Binding of Hsp70 stimulates an endogenous ATPase, which causes the release of bound protein. How is such preferential binding of unfolded molecules accomplished? The Hsp70s recognize a feature common to the denatured, but not the native, states of proteins: an exposed, extended region of polypeptide rich in hydrophobic residues (*3–5*). This recognition of hydrophobic peptides, which is relatively sequence-independent, could be accomplished by way of a binding site similar to that of the peptide binding domain of the major histocompatibility complex class I molecule (*6*).

Although the binding of an Hsp70 chaperone may keep a partially folded protein from aggregating, the same interactions will also block folding because the hydrophobic regions must be buried in the fully folded molecule. Completion of folding requires the release of the protein from the Hsp70 chaperone, at which point a partially folded protein is again vulnerable to aggregation. Indeed, rather than promote folding to the native

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