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Protein Folding In Vivo: Unraveling Complex Pathways

Minireview

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Folding of proteins in a test tube is a private affair. Protein folding in a cell is another matter entirely. In the complex milieu of the cell, the conditions of temperature, pH, and ionic strength are restricted, and the concentration of macromolecules is high. Folding in this environment is thought to be facilitated by molecular chaperones, a class of proteins that bind to unfolded or partially folded polypeptides (reviewed by Hartl, 1996). As unfolded polypeptides contain many more exposed hydrophobic residues than do polypeptides in their native state, they are exquisitely susceptible to aggregation. Whether the polypeptide is a nascent chain on a ribosome or a mature protein recently unfolded due to stress, suppression of aggregation is essential in order to maintain proteins in a state competent for folding. Chaperones bind to hydrophobic regions of nonnative proteins, hindering aggregation. Therefore, through regulated cycles of peptide binding and release, chaperones facilitate the acquisition of the active conformation of polypeptides. Distributed throughout the cell, molecular chaperones have the opportunity to act at different periods of a protein's lifetime to assist in folding during and immediately after translation, translocation across membranes, or refolding after denaturation caused by stress. Two lines of evidence led to the idea that molecular chaperones facilitate the folding of proteins in the cell. First, certain newly synthesized proteins in the cell transiently associate with molecular chaperones. Secondly, molecular chaperones can facilitate the folding of certain proteins in vitro. However, the question remains: What is the role of molecular chaperones in the folding of the wide array of proteins synthesized in the cell?

The Establishment: Hsp70/DnaJ and Chaperonin Classes of Chaperones

Two classes of molecular chaperones have been extensively studied, the Hsp70s and the chaperonins. The Hsp70s are ubiquitous proteins found in the E. coli cytosol and several compartments of eukaryotic cells including the ER lumen, the mitochondrial matrix, and the cytosol. The cyclic interaction of Hsp70 with a polypeptide is regulated by the binding of adenine nucleotides to its N-terminal ATPase domain. Hsp70s' actions are facilitated by cochaperones such as members of the DnaJ family. DnaJs are thought to work together with Hsp70s by initially binding nonnative proteins, by targeting them to Hsp70, and by stimulating Hsp70's ATPase activity. Chaperonins, a class of chaperones whose role in protein folding in vitro has been extensively analyzed, include the well-studied GroEL/ES oligomeric complex of bacteria, as well as the closely related Hsp60/Hsp10 complexes of mitochondria and chloroplasts, and the more distantly related CCT/TriC complex of the eukaryotic cytosol. The GroEL/ES prototype, composed of a heptameric GroEL ring with a heptameric GroES cap, forms a cage in which bound polypeptides may reach their native state. The release of GroES coupled to ATP binding and hydrolysis by GroEL allows cycles of release of folded and partially folded polypeptides and rebinding of nonnative species in the central cavity of GroEL (reviewed by Fenton and Horwich, 1997).

For a number of years the predominant model for an in vivo protein folding pathway has been the sequential action of Hsp70s (with DnaJs) and chaperonins in which the substrate interacts first with Hsp70 and then with chaperonins (Figure 1, lower pathway). This idea stemmed from the identification of such a pathway in mitochondria. Since mitochondrial Hsp70 is a critical component of the translocation machinery, binding polypeptides as they enter the matrix, it is positioned to bind incoming polypeptides prior to Hsp60, the mitochondrial chaperonin. In the cytosol, targeting of Hsp70 to translating ribosomes (James et al., 1997) may also preferentially allow Hsp70 binding to nascent chains prior to CCT/TriC binding. In vitro evidence supports transfer of some substrates from Hsp70s to chaperonins; Hsp70/DnaJ can bind denatured rhodanase, preventing its aggregation and maintaining it in a "folding competent" state until addition of GroEL/ES, which facilitates efficient acquisition of the native state (reviewed by Hartl, 1996). Such a pathway seems reasonable, since Hsp70s appear to bind preferentially peptides lacking secondary structure, while chaperonins interact with partially folded intermediates. However, reciprocal transfer of denatured firefly luciferase back and forth between the bacterial Hsp70/DnaJ and GroEL/GroES chaperones was recently demonstrated, suggesting that protein folding pathways are not necessarily unidirectional (Buchberger et al., 1996). In fact, GroEL is capable of preventing aggregation of unfolded firefly luciferase and maintaining it in a state capable of refolding by the Hsp70 system at a later time. Together, these results suggest that the apparent pathway revealed by in vitro experiments may depend on the order of addition of chaperone components as well as the test polypeptide utilized. In the cell, a polypeptide released from one class of chaperone may undergo kinetic partitioning among a number of chaperones present within the same cellular compartment, rather than relying solely on a single unidirectional pathway.

An Hsp70/chaperonin pathway may serve a general role in protein folding in the E. coli cytoplasm and in mitochondria, as both types of chaperones are abundant in these cellular compartments. However, the chaperonin of the eukaryotic cytosol, CCT/TriC, is of much lower abundance in many cell types, certainly at a level inadequate for handling the folding of the bulk of cytosolic proteins. In vitro and in vivo evidence indicates a role for CCT/TriC in the maturation of components of the cytoskeleton such as actin and tubulin (reviewed by Hartl, 1996). Even though in reticulocyte lysates both Hsp70 and CCT/TriC are needed for efficient cotranslational folding of firefly luciferase (Frydman et al., 1994),

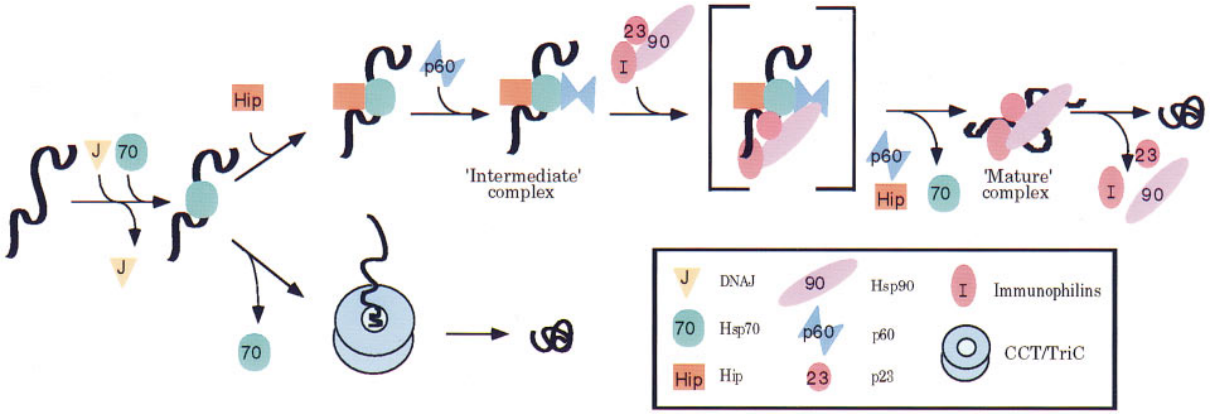

Figure 1. Folding Pathways of the Eukaryotic Cytosol

Two eukaryotic cytosolic chaperone systems are described in the text. Although presented as unidirectional pathways, reciprocal transfer between different chaperone components is likely in vivo. Some substrate specificity appears to exist in vivo, as steroid receptors and oncogenic protein kinases use the Hsp90 pathway, while others, such as actin and tubulin rely on the CCT/TRiC pathway. Whether a substrate polypeptide folds independently of chaperones or uses only a subset of those chaperones available probably depends mostly on the structural state of the protein itself and the general availability of chaperones.

the action of this chaperonin is most likely limited to a small subset of proteins in vivo due to its low abundance. Therefore, we are faced with the question of the role of chaperones in the folding of most eukaryotic cytosolic proteins. The abundant cytosolic Hsp70s are obvious candidates for such a role because they are able to facilitate folding of the denatured test substrates E. coli β-galactosidase in purified systems and firefly luciferase in both purfied systems and cell lysates. However, although there is substantial evidence for a role of these proteins in binding to nascent chains on ribosomes, regulating the heat shock response, and facilitating translocation of at least some proteins from the cytosol into organelles, there is as yet no in vivo evidence for a direct role in protein folding.

Hsp90, a Possible General Cytosolic Chaperone

What protein(s), besides those of the CCT/TriC system, could handle the folding needs of cytosolic proteins along with Hsp70s? Recently, a number of papers have suggested a role of Hsp90 and its partner proteins in the folding of cytosolic proteins. Hsp90 is an abundant, highly conserved protein best known for its role in maturation of steroid receptors and oncogenic protein kinases such as pp60 v-Src (v-Src). Hsp90 appears unable to facilitate refolding of denatured substrates on its own, and thus does not fit the classical definition of a molecular chaperone. However, Hsp90 does satisfy a currently utilized, albeit limited, working definition of molecular chaperones in that it is able to suppress the aggregation of several unfolded proteins, including casein kinase II and citrate synthase (Bose et al., 1996). Additionally, Hsp90 is able to maintain some denatured proteins in a state competent for refolding after addition of Hsp70 and a DnaJ homolog, suggesting the possibility that Hsp90 may act in a complex folding pathway (Freeman and Morimoto, 1996).

Proteins of the Hsp90–Receptor Complexes

An early clue to the potentially elaborate nature of in vivo Hsp90 protein folding pathways originated from studies that identified a group of proteins associated with inactive steroid hormone receptors and oncogenic protein kinases. As progesterone receptor matures in a reticulocyte lysate system, it can be found in complexes involving at least nine different proteins: Hsp90, Hsp70, Hip, p60, p23, a DnaJ homolog, and the immunophilins FKBP52, FKBP51, and CyP-40 (Table 1). These proteins, which dissociate from the receptor upon hormone binding, are now thought to play a role in forming the active state of the receptor (Pratt et al., 1996). Evidence for the importance of these proteins in receptor and kinase maturation in vivo comes from two types of analyses: genetic experiments in S. cerevisiae and inhibitor studies in vertebrate cells. In yeast, maturation of heterologous steroid receptors or of v-Src to an active state requires Hsp90, Sti1 (the p60 homolog), Ydj1 (a DnaJ homolog), and Cpr7 (a Cyp-40 homolog) (reviewed by Bohen et al., 1995; Duina et al., 1996; Chang et al., 1997). These proteins are linked physically as well as functionally; Hsp90 isolated from yeast is associated with an array of proteins similar to that found in reticulocyte lysate: Hsp70 of the *SSA* family, Sti1 (the p60 homolog), and two Cyp40-homologs, Cpr6 and Cpr7. In intact vertebrate cells, the benzoquinoid ansamycins geldanamycin (GA) and herbimycin A have proven to be valuable

Table 1. Proteins of the Hsp70/Hsp90 Folding Pathway

Vertebrate	S. cerevisiae	Comments
Hsp90	Hsc82/Hsp82	chaperone
Hsp70	SSA family	chaperone
p60/Hop/IEFSSP3521	Sti1	binds Hsp90, Hsp70
Hip/p48	?[1]	binds ADP form of Hsp70
p23	?[1]	chaperone, binds Hsp90
FKBP52/hsp56/p59	?[1]	chaperone, binds Hsp90
FKBP51	?[1]	binds Hsp90
CyP-40	Cpr6, Cpr7	chaperone, binds Hsp90
Hdj-1, Hdj-2	Ydj1	chaperone, binds Hsp70

[1]A p23-related sequence exists in the Saccharomyces Genome Database. No sequences clearly related to these large FKBPs or Hip/p48 are present (http://genome.www.stanford.edu/Saccharomyces/).

tools for studying Hsp90 function. These compounds bind Hsp90 and disrupt its interactions with target proteins. Long known to revert the growth phenotype of v-Src-transformed cells, they also inhibit glucocorticoid and progesterone receptor activation in vivo (Nair et al., 1996, and references therein).

If the other proteins of the Hsp90 complex function in the formation of active receptor, they may have molecular chaperone activity themselves or act as modulators of Hsp70 and Hsp90. Recently, p23, FKBP52, and Cyp-40 have been shown to bind at least some unfolded polypeptides, suggesting they have some properties of molecular chaperones. Freeman et al. (1996) found that, while neither p23 nor Cyp-40 could facilitate the folding of substrate on its own, either protein was able to maintain denatured β-galactosidase in a state competent for folding. Independently, Bose et al. (1996) found that either p23 or FKBP52 was able to suppress the aggregation of citrate synthase. The role of the peptidyl-prolyl isomerase activity in the functioning of the immunophilins remains unknown, as the presence of the immunosuppressant drugs, which bind and inhibit the peptidyl-prolyl isomerase activity, had no effect on the chaperone properties of CyP-40 and FKBP52 in these in vitro folding assays. However, while p23, FKBP52, and Cyp40 have some properties of chaperones in vitro, the nature, as well as the importance, of binding of these proteins to natural substrates in vivo remains to be analyzed.

Two of the other components appear to have important roles in modulating the activity of chaperones. Hip, like DnaJ, regulates Hsp70 activity by controlling whether Hsp70 is bound predominantly to ADP or ATP. The ATP-bound form of Hsp70 rapidly binds and releases polypeptide; in contrast, the ADP-bound form has slower on and off rates. Thus, hydrolysis of ATP to ADP by the intrinsic Hsp70 ATPase, which is stimulated by DnaJ, results in a stabilization of the interaction with a substrate polypeptide. Hip binds preferentially to the ADP-bound form of Hsp70, slowing the release of nucleotide (reviewed by Ziegelhoffer et al., 1996). Consequently, Hip and DnaJ stabilize the Hsp70–substrate polypeptide interaction. Exchange of ATP for ADP probably effects the release of polypeptide, thus completing a cycle of peptide binding and release.

p60 is the only protein in the receptor complex for which neither chaperone nor modulator activity has been found. It is also the only protein of this pathway that is able to bind both Hsp90 and Hsp70. Complexes between purified Hsp90 and Hsp70 form only in the presence of p60, suggesting the possibility that it may act as a physical "connector" between the two chaperones (Johnson et al., 1996).

An Hsp70/Hsp90 Folding Pathway?

A complex of Hsp90, p23, and the immunophilins can form in the absence of substrate (Johnson et al., 1996). Like the chaperonins, Hsp90 appears to bind substrates possessing secondary and tertiary structure (Jakob et al., 1995). These two findings, along with recent data on steroid receptor maturation, suggest that a folding pathway involving transfer of substrates from Hsp70 to this Hsp90–p23–immunophilin complex might exist. The following sequence of events may occur in such a pathway (Figure 1, upper pathway): in an initial step, which may be dependent on a DnaJ homolog, Hsp70 binds the receptor. DnaJ stimulates the ATPase activity of Hsp70, thus converting Hsp70 to the ADP-bound form, which is then stabilized by the binding of Hip. Together, these proteins act to stabilize the Hsp70–substrate polypeptide complex. Subsequent binding of Hsp70 by p60, which is also capable of binding Hsp90, serves to recruit Hsp90 and associated chaperones, p23 and immunophilins, to the substrate. Binding of ATP to Hsp70 causes destabilization of the Hsp70–substrate interaction and the release of Hsp70, Hip, and p60 from the complex. As a final step, Hsp90, p23, and immunophilins facilitate the formation of the active receptor capable of hormone binding.

Evidence for this model of an Hsp70/Hsp90 pathway of folding comes from studies of steroid receptor maturation (reviewed by Pratt et al., 1996; Nair et al., 1996) in reticulocyte lysate. When ATP is limited, the progesterone receptor becomes trapped in an "intermediate" form, characterized by Hsp70, p60, Hip, and substochiometric amounts of Hsp90. ATP addition is required for binding of the Hsp90–p23–immunophilin complex to the substrate, resulting in the "mature" progesterone receptor complex. The ability of the Hsp90 inhibitor GA to trap the intermediate receptor complex, preventing association of the Hsp90–p23–immunophilin complex, provides evidence for the movement of the receptor from one complex to another.

Implications from the in vivo studies examining the role of Hsp90 and partner proteins parallel those from in vitro studies. Deletions or mutations in the genes encoding Hsp90, Ydj1, Cpr7, and Sti1, homologs of four of the nine proteins identified in Hsp90–substrate complexes in vitro, decrease the activity of v-src and steroid receptors expressed in S. cerevisiae. However, the role of other components in vivo is unknown, as homologs of Hip, p23, and the FKBPs have not been described in yeast (Table 1). Proteins yet to be identified, either in vertebrate or yeast systems, may also function in this pathway. For example, while p60 seems critical for the association of purified Hsp90 and Hsp70, Hsp90 complexes isolated from yeast lacking Sti1, the yeast homolog of p60, still contain Hsp70 (Chang et al., 1997), suggesting other proteins may be able to mediate an interaction between Hsp70 and Hsp90. More differences between the yeast and mammalian studies may emerge, due either to innate differences between the organisms or experimental differences between the approaches used.

The Repertoire: More than Receptors and Kinases?

As described above, abundant evidence exists for a critical role of Hsp90 and its partner proteins in the maturation of a variety of kinases, in addition to several steroid hormone receptors in mammalian cells (Nair et al., 1996). However, no native yeast substrates have been identified, despite evidence that Hsp90, Sti1, Ydj1, and Cpr7 are involved in the maturation of heterologous receptors and kinases. Yet, Hsp90 is essential for viability and combinations of mutations in genes encoding Sti1, Hsp90, and Cpr7 cause synthetic growth defects (Duina et al., 1996; Chang et al., 1997), suggesting important roles for these complexes. But whether a Hsp70/

Hsp90 pathway has a general function to facilitate folding of many newly synthesized proteins or to refold proteins after denaturation in response to stress remains to be determined. At this time, we know that GA inhibits the refolding of firefly luciferase expressed in vertebrate cells after heat shock (Schneider et al., 1996), as well as the rate of thermally denatured luciferase renaturation in reticulocyte lysate. Furthermore, heat-denatured luciferase added to reticulocyte lysate can be coimmunoprecipitated with Hsp90, Hsp70, p60, Hip, and p23, thus suggesting a role for this entire complex of proteins in this folding process (Schumacher et al., 1996; Thulasiraman and Matts, 1996). However, it should be kept in mind that only a single substrate has been analyzed in these studies and that this protein is normally resident in the peroxisome, not the cytosol.

Future Perspectives

The results considered in this review provide a glimpse of the probable complexity of protein folding as it occurs in the eukaryotic cytosol in vivo. Multiple chaperones may exist within a complex, suggesting a dynamic interaction in which more than one chaperone simultaneously binds a substrate polypeptide during the process of folding. The reciprocal transfer back and forth between the bacterial Hsp70/DnaJ and GroEL/GroES chaperones suggests protein folding pathways need not be unidirectional (Buchberger et al., 1996). Similar reciprocal movement along an Hsp70/Hsp90 pathway is likely, with the possibility of a multitude of different chaperone complexes. Perhaps, rather than using obligate sequential pathways, substrates in vivo move back and forth between a network of chaperone systems depending on their structural state and the availability of the different chaperones, with some interactions being productive and leading towards the native state.

Over the past decade, the pendulum has swung from the norm being disbelief in molecular chaperones as important in protein maturation in vivo, toward a view that molecular chaperones are required for each and every step of the life of each and every protein. Our knowledge of the variety and mechanism of action of molecular chaperones has advanced sufficiently that the time has come to step back and begin to dissect the role of chaperones in the folding of polypeptide substrates in their natural environment. Although experimentally challenging, the reward will be a more realistic view of protein folding in the complex cellular environment.

Selected Reading

Bohen, S., Kralli, A., and Yamamoto, K. (1995). Science *268*, 1303–1305.

Bose, S., Weikl, T., Bügl, H., and Buchner, J. (1996). Science *274*, 1715–1717.

Buchberger, A., Schroder, H., Hesterkamp, T., Schonfeld, H., and Bukau, B. (1996). J. Mol. Biol. *261*, 328–333.

Chang, H., Nathan, D., and Lindquist, S. (1997). Mol. Cell. Biol. *17*, 318–325.

Duina, A., Chang, H., Marsh, J., Lindquist, S., and Gaber, R. (1996). Science *274*, 1713–1715.

Fenton, W., and Horwich, A. (1997). Protein Science *6*, 743–760.

Freeman, B., and Morimoto, R. (1996). EMBO J. *15*, 2969–2979.

Freeman, B., Toft, D., and Morimoto, R. (1996). Science *274*, 1718–1720.

Frydman, J., Nimmesgern, E., Ohtsuka, K., and Hartl, F. (1994). Nature *370*, 111–117.

Hartl, F. (1996). Nature *381*, 571–580.

Jakob, U., Lilie, H., Meyer, I., and Buchner, J. (1995). J. Biol. Chem. *270*, 7288–7294.

James, P., Pfund, C., and Craig, E. (1997). Science *275*, 387–389.

Johnson, J., Corbisier, R., Stensgard, B., and Toft, D. (1996). J. Steroid Biochem. Mol. Biol. *56*, 31–37.

Nair, S., Toran, E., Rimerman, R., Hjermstad, S., Smithgall, T., and Smith, D. (1996). Cell Stress Chaperones *1*, 237–250.

Pratt, W., Gehring, U., and Toft, D. (1996). In Stress-Inducible Cellular Responses, U. Feige, R. Morimoto, I. Yahara, and B. Polla, eds. (Basel: Birhauser Verlag) pp. 79–95.

Schneider, C., Sepp-Lorenzino, L., Nimmesgern, E., Ouerfelli, O., Danishefsky, S., Rosen, N., and Hartl, F. (1996). Proc. Natl. Acad. Sci. USA *93*, 14536–14541.

Schumacher, R., Hansen, W., Freeman, B., Alnemri, E., Litwack, G., and Toft, D. (1996). Biochemistry *35*, 14889–14898.

Thulasiraman, V., and Matts, R. (1996). Biochemistry *35*, 13443–13450.

Ziegelhoffer, T., Johnson, J., and Craig, E. (1996). Current Biol. *6*, 272–275.