[Frontiers in Bioscience 1, d309-317, 1 October 1996]

HSP90 - NEWS FROM THE FRONT

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1. ABSTRACT

The 90 kDa heat shock protein Hsp90 is a highly conserved and very abundant protein in the cytosol of both eukaryotic and prokaryotic cells. The main focus in the recent years has been concerned Hsp90's interaction with untransformed steroid receptors and newly synthesized kinases. Within these heterocomplexes, Hsp90 acts in concert with several other heat shock and non heat shock proteins to mediate important regulatory effects. These roles of Hsp90 leave unexplained its high abundance and heat shock regulation. More recently, however, Hsp90 has been identified as an ATP independent molecular chaperone, which binds transiently to folding intermediates *in vitro*, prevents aggregation and supports the refolding of the intermediates to the native state. The finding that Hsp90 interacts with late, probably highly structured, folding intermediates led to the suggestion that Hsp90 might function as a general chaperone for well structured not yet native polypeptides. This explanation provides the missing link between Hsp90 on the one hand as a highly specialized binding protein and Hsp90 on the other hand as a rather promiscuous molecular chaperone.

2. HEAT SHOCK PROTEINS AND MOLECULAR CHAPERONES - A HISTORICAL INTRODUCTION

It was more than 30 years ago, when Ritossa and coworkers found that an increase in incubation temperature of *Drosophila melanogaster* larva results in the development of a defined set of new transcription loci on the polytene chromosome (1). Not many people took notice of this important discovery, and it was not until more than 12 years later that the first gene products were identified and the term "heat shock protein" (Hsp) was coined (2). In the following years the heat shock proteins were discovered to be present in all species tested and to be extremely well conserved (reviewed in ref. 3). Even thermophilic organisms, which optimal growth temperature lies between 50° and 90° C have been found to respond sudden temperature upshifts with the overexpression of heat shock proteins. However, while the regulation of heat shock proteins was studied extensively, relatively little was known about their physiological significance (3). It needed the combinational work of many labs to put the pieces together and to revolutionize the protein folding field. The first hint came well before the characterization of heat shock proteins in *E. coli* with the work of Georgopoulos and collaborators, who discovered that successful lambda phage head assembly depends on the presence of a host-produced protein, subsequently called GroE (4). A few years later, the same group of scientists found another host gene, *dnaK*, which was shown to be involved in lambda replication (5). That GroE mediated protein folding and assembly is not restricted to phage assembly and DnaK mediated disruption of certain protein-protein interactions is not limited to phage replication (6) became evident with the identification and functional characterization of their eukaryotic homologues. Rubisco binding protein, a chloroplast protein implicated in the assembly of the ribulose 1,5 bisphosphate carboxylase (rubisco) multimeric complex (7) was shown to be closely related to GroEL (8) and the mammalian clathrin uncoating ATPase was identified as Hsc70, the eukaryotic DnaK homologue (9). This was the beginning of the chaperone era and the end of

Received: 9/9/96; Accepted: 9/10/96

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the idea that all proteins reach their specific three dimensional structure spontaneously in the cell (10).

The discovery of folding helper proteins (chaperones) did not contradict Anfinsen's classic theory about protein folding which states that the aquisitation of the specific three dimensional protein structure depends exclusively on the amino acid sequence of the individual protein (11). Chaperones do not change the path of protein folding or the final fold. However, they do keep proteins on the correct folding pathway and prevent unspecific interactions (12), therefore regulating the kinetic competition between folding, specific association and non-specific, irreversible aggregation (13). Aggregation represents a major problem for a folding protein. Since it is a second or higher order reaction it can often be circumvented *in vitro* by decreasing the protein concentration (14). Cells, however, have a very high concentration of aggregation sensitive folding intermediates at any specific time in a predefined "reaction volume" (15), and therefore need the help of chaperones. Chaperones bind transiently to folding intermediates, keep their free concentration low and suppress otherwise fatal interactions (16).

Aggregation-prone folding intermediates are not unique to newly synthesized polypeptides, but are formed in even higher concentration as a result to dramatic environmental changes such as heat shock and viral infections (17, 18). This explains the high constitutive expression level of heat shock proteins under normal temperature conditions as well as their several-fold overexpression under heat shock conditions (reviewed by 19, 20).

The most prominent heat shock proteins which take care of otherwise irreversibly damaged proteins in eukaryotes are members of the Hsp90 and Hsp70 families. While Hsp70 was one of the first proteins known to function as a molecular chaperone, the function of Hsp90 both under normal and stress conditions has remained elusive. Only very recently, a substantial increase in the understanding has been reported (reviewed in ref. 21).

3. DISCUSSION

3.1. Hsp90

Until only a few years ago, work on Hsp90 was mainly focused on its interaction with untransformed steroid receptor complexes and newly synthesized tyrosin kinase complexes (reviewed in refs. 22, 21). These specific interactions, however, could not explain why Hsp90 i) represents the most abundant heat shock protein under normal conditions and therefore exceeds the concentration of steroid receptors and kinases by about 1000 fold (23) ii) why it is dramatically upregulated by various conditions such as heat shock (3), iii) why it is essential in yeast (24) and iv) and why it is well conserved in organisms such as prokaryotes, cell compartments such as the endoplasmic reticulum and organelles such as the chloroplasts, where the known substrate proteins are not present (25-27). It has now become clear that Hsp90 functions as a general molecular chaperone *in vitro* and *in vivo*, acts in concert with many other heat shock and non heat shock proteins and shows a much broader substrate specificity than originally assumed.

3.2. Hsp90 - A specific binding protein *in vivo*

In 1986, two groups independently reported the existence of a non-receptor protein in untransformed steroid receptor complexes (28, 29). This protein was shown to be the dimeric, 90 kDa heat shock protein Hsp90. Following studies showed, that interaction of Hsp90 with hormone free steroid receptors plays a central role in receptor regulation and activation (reviewed by 22, 30). However, it is now clear that Hsp90 is not the only non receptor protein within these heterocomplexes. Hsp90 has been found to be associated with at least 7 other partner proteins in hormone free receptor heterocomplexes (21, 22, 31-33). This complex formation is the prerequisite for hormone binding. After binding of the ligand and dissociation of the heterocomplex into the individual proteins, the hormone bound receptor undergoes various modifications and interactions, which finally lead to its activation as transcription factor. Central to its role in receptor regulation is the interaction of Hsp90 with the signal domain (hormone binding domain) of the aporeceptor (34). This domain is responsible for hormone binding in the presence of ligands as well as for the inactivation of the DNA binding domain of the receptor in the absence of ligands (35). Based on *in vitro* experiments it seems reasonable to conclude that the function of Hsp90 is not simply to mediate the inactivation of steroid-receptors in the absence of the ligand but rather to keep receptors in an activatable state (30). The interaction of Hsp90 with hormone free receptors induces the formation of a high affinity ligand binding conformation within the hormone binding domain and is also important for subsequent maintenance of this conformation (30). Conformational changes between the hormone and DNA binding domain of the receptor upon Hsp90 association and dissociation have been demonstrated (36).

Since the additional proteins of the heterocomplex have been identified only very recently, little is known about their role in receptor regulation (31, 32). To elucidate their function, cell-free systems have been established to study the influence of the various members on successful

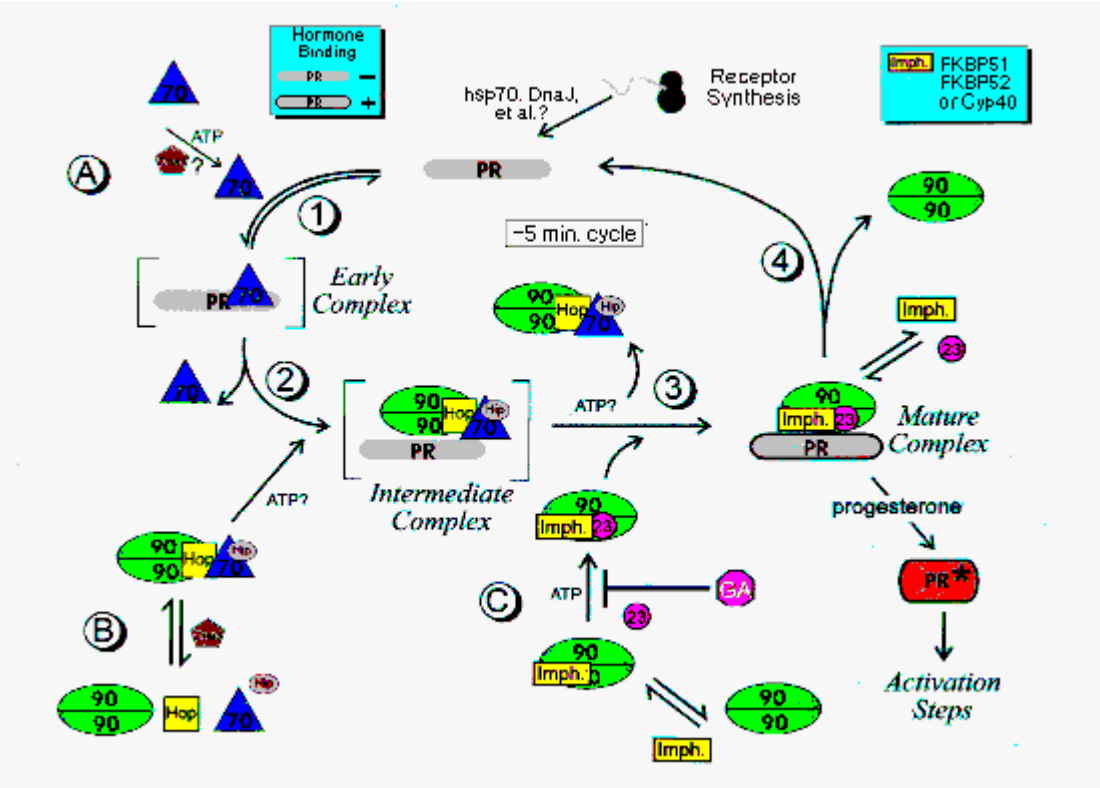

Figure 1: Working model for the dynamic and transient interactions of progesterone receptor with the Hsp90 superchaperone complex (adapted from ref. 32). Figure courtesy of David Smith. Step 1: ATP-dependent formation of an early complex between Hsp70 and either newly synthesized or folded progesterone receptor (PR). Step 2: Formation of an intermediate complex between PR and a preformed complex consisting of Hsp90, Hop, Hsp70 and Hip. It is still unclear at what time and stage Hip enters the complex and whether the formation of the early complex is a necessary prerequisite for step 2. Step 3: Formation of the mature complex between PR and a preformed complex of Hsp90, immunophilin (either one of the three immunophilins FKBP54, FKBP52 or Cyp40) and p23. The PR is now active (indicated by the solid black line). After dissociation of the complex, PR can either bind hormone and undergo activation as transcription factor, or in the absence of ligands interact with Hsp70 to start the new cycle over again.

heterocomplex assembly. In the case of the progesterone receptor, Hsp90 and at least 7 other proteins are involved (32): Hsp70, Hip, Hop, immunophilins and p23. Hsp70 promotes protein folding and might "prepare" the conformation of steroid-receptors for Hsp90 binding (37). Hip (Hsp70 interacting protein), also called p48, seems to regulate the ATPase activity of Hsp70 (38). The role of Hop (Hsp70-Hsp90 organizing protein), better known as p60, is still very much unknown, except that it has been found in complex with Hsp90, Hsp70 and Hip also in the absence of untransformed receptors (39). Another group of proteins involved in heterocomplex assembly are the three immunophilins FKBP54, FKBP52 (Hsp56, p59) and Cyp40. Since all three isomerases compete for the single common immunophilin binding site on Hsp90, only one of them is found in any given heterocomplex but all three of them are present in heterocomplex preparations (40, 41). Curiously, peptidyl prolyl isomerase activity appears not to be required for complex formation since the presence of the respective immunosuppressor does not interfere with the complex formation (22). Finally, an unique protein called p23 copurifies with Hsp90 from all tissues tested and can be reconstituted into a functional complex with Hsp90 and immunophilins *in vitro* in an ATP-dependent manner (42, 43). The origin of the ATP-dependence is still unclear, but transient involvement of Hsp70 seems a reasonable explanation.

The discovery that Geldanamycin specifically disrupts certain interactions within the Hsp90 complex (44) led to a detailed working model about the series of events taking place during the superchaperone-progesterone receptor heterocomplex assembly (see Figure 1) (adapted from ref. 32).

The second group of proteins, with which Hsp90 forms stable complexes are certain viral and cellular protein kinases. Hsp90 has been shown to associate with newly synthesized, possibly aggregation sensitive tyrosine kinases until the kinase becomes attached to the plasma membrane (45, 46). Hsp90 clearly plays an important role in the maturation of these kinases. In this context it has been demonstrated that Hsp90 and the partner protein p50 form stable complexes with temperature sensitive $pp60^{v\text{-}src}$ mutants under non-permissive temperatures. Temperature shifts into the permissive range result in the dissociation of the complex and insertion of $pp60^{v\text{-}src}$ into the plasma membrane (47). Furthermore it has been shown, that the number of active, membrane associated $pp60^{v\text{-}src}$ molecules is lower in cells expressing low levels of Hsp90 (48). And recently it has been demonstrated that benzochinon ansamycins like Geldanamycin (GA) result in lower levels of active tyrosine kinases since the interaction of GA with Hsp90 leads to the disruption of Hsp90-$pp60^{v\text{-}src}$ complexes. This appears to result in an increased proteolytic turnover of $pp60^{v\text{-}src}$ (32,44).

The observation that Hsp90 interacts with $pp60^{v\text{-}src}$ but not with the closely related cellular homologue $pp60^{v\text{-}src}$ resulted in the speculation that the substrate specificity of Hsp90 is rather narrow (48). However, only recently it has been demonstrated, that Hsp90 is able to interact with both viral and cellular members of certain tyrosin kinases (49). This makes it more reasonable to assume, that differences in interactions with Hsp90 are based on differences in the folding pathway and stability of the respective kinases rather then on the substrate specificity of Hsp90 (21, 49).

The partner proteins of Hsp90 which are present in receptor complexes have also been identified as members of Hsp90-protein kinase complexes, as well as members of heat shock factor 1-Hsp90 complexes (33, 50), suggesting that they may play a general role in Hsp90 function. The function of p50, a protein, which represents the first partner protein of Hsp90 identified (45) and which has so far only been detected in Hsp90-kinase complexes remains to be established.

3.3. Hsp90 - A folding helper protein *in vitro*

Chaperones function by their ability to recognize and transiently bind aggregation-prone folding intermediates, therefore suppressing non-specific aggregation and increasing the yield of proper folded proteins (16). The chaperone activity of proteins has been investigated *in vitro* by studying their influence on substrate proteins which are unable to refold after denaturation due to aggregation processes. Figure 2 shows the influence of Hsp90 on the refolding of chemically denatured citrate

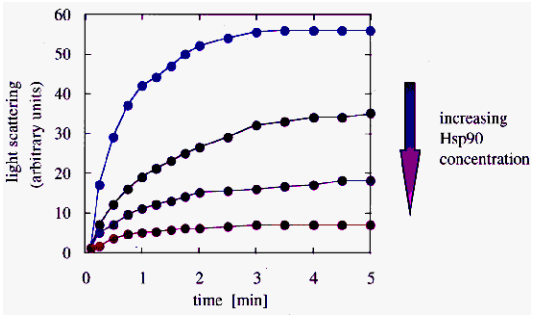

Figure 2: Influence of Hsp90 on the aggregation of refolding citrate synthase (51).

synthase, a chaperone model substrate, by monitoring aggregation (51).

Denatured citrate synthase in the absence of added chaperones spontaneously aggregates upon dilution into renaturation buffer (52). Within a few seconds, light scattering can be detected. In the presence of stoichiometric amounts of Hsp90, aggregation is significantly suppressed, suggesting that Hsp90 binds to these aggregation sensitive folding intermediates. Therefore, Hsp90 partitions a larger number of molecules into a productive folding pathway, thus allowing a higher number of molecules to reach the active, native state. Hsp90 influences the folding and activity of a number of different non-native proteins, suggesting that Hsp90 functions *in vitro* as a molecular chaperone with rather broad substrate specificity. These studies included the finding that Hsp90 is able to chaperone casein kinase II by preventing unproductive aggregation (53) and that the presence of Hsp90 or equally well just a C-terminal fragment of Hsp90 resulted in twofold more active muscle specific protein MyoD (54, 55). In none of these cases has a stable interaction between isolated Hsp90 and its substrate proteins been detected. The folding kinetics of citrate synthase in the presence of Hsp90 instead suggested that reactivation of the enzyme occurs via successive binding-release-rebinding cycles with folding intermediates. After the release from Hsp90, the intermediates face at least three possible routes (Figure 3): they can either refold to the native state, being therefore no longer substrate of Hsp90, rebind to Hsp90 or interact with other folding intermediates to form aggregates (21). Which route they take will depend on the microscopic rate constants of folding and association as well as on the concentration of folding intermediates and Hsp90. To favor rebinding to Hsp90 and prevent aggregation, an excess of Hsp90 is required. This excess is guaranteed by the high abundance of Hsp90 in the cell (51). In the case of citrate synthase, casein kinase and MyoD transient interactions with Hsp90 are sufficient to promote proper refolding. β-galactosidase, however, seems to need the combined action of Hsp90, Hsp70 and Hdj-1

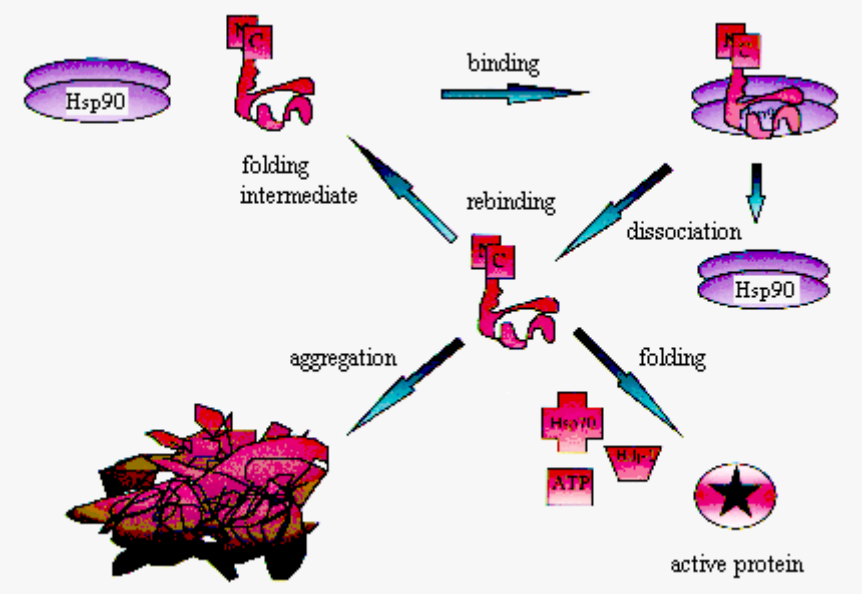

Figure 3: Working model for the interaction of Hsp90 with unfolded substrate proteins.

to regain activity (56). Folding conditions, folding rates and the nature of the folding intermediates probably determine the need of specific folding helper proteins. Under permissive folding conditions as in the case for citrate synthase, folding may take place simply after the release of Hsp90. Rebinding to Hsp90 will be only achieved using high amounts of Hsp90 (51). Under less permissive folding conditions, however, where spontaneous folding rates are comparatively slow, refolding of the enzyme becomes outcompeted by the rebinding to Hsp90 and the protein appears to be stably associated with Hsp90 in a folding competent state. This might be the case for β-galactosidase, where the ATP-dependent chaperone activity of Hsp70 and Hdj-is required to support the functional refolding of the enzyme. According to this model, the functional mechanism of Hsp90 is very similar to the mechanism described for the GroE system (summarized in ref. 23). Under permissive folding conditions, the presence of GroEL alone is often sufficient to promote refolding of certain substrate protein by iterative cycles of binding, release and rebinding (57-59). Under suboptimal or non permissive conditions, however, the additional help of either ATP or ATP and GroES for efficient refolding is required, otherwise the substrate protein stays associated with GroEL in an apparently stable complex maintained in a folding competent state (60). The role that ATP plays in this reaction was recently illuminated by showing that ATP decreases the on-rate of rebinding of the substrate protein to GroEL by three orders of magnitude (61). The prolonged time in which the folding intermediate is not associated with GroEL allows refolding to occur under conditions where the folding rate of the substrate protein is rather slow. Under non permissive conditions, however, where spontaneous refolding is unlikely, the additional presence of GroES is required (summarized in ref. 23).

For some time the ATP independence of Hsp90 action *in vitro* has been controversial (21). Two groups have reported ATP binding and ATPase activity of Hsp90 (62-65). However, careful use of a number of techniques with the appropriate controls has now ruled out ATP binding or ATPase activity for Hsp90 (66). It remains possible that proteins associated with Hsp90 may have these activities.

3.4. Hsp90 - A heat shock protein

Hsp90 is a very abundant protein under normal conditions, but its concentration increases even more upon sudden temperature upshifts (3). Although an important fact, only very few *in vivo* approaches have been undertaken to study the function of Hsp90 under heat shock conditions in the cell. It has been demonstrated, that i) deleting *E. coli* Hsp90 results in a slight growth disadvantage at elevated temperatures (67), ii) that yeast Hsp90 is essential at any temperature (24) and iii) that decreasing the high intracellular Hsp90 concentration leads to an increased mortality of mammalian cells at elevated temperatures (68). To gain more insight in the protective role Hsp90 plays under conditions where unfolding and subsequently aggregation of polypeptides occurs, the influence of isolated Hsp90 on thermally unfolding proteins has been studied. In these *in vitro* studies it has been shown, that both eu- and prokaryotic Hsp90 binds transiently to thermally

unfolding intermediates of the model substrate citrate synthase, thereby apparently stabilizing the enzyme and suppressing aggregation (69, 70). In the presence of Hsp90, folding intermediates are kept significantly longer in a folding competent state (69). Experiments which study the refolding of thermally inactivated firefly luciferase revealed the additional need for Hsp70 and ATP for efficient refolding of luciferase (71) similar to what was seen with chemically unfolded β-galactosidase. It is likely to assume, that Hsp90 keeps luciferase in a folding competent state, while Hsp70 and ATP promotes the functional renaturation of luciferase. Taken together these findings suggest that Hsp90 plays a general role in protein folding and assembly processes. Depending on the substrate protein, Hsp90 might act in conjunction with other chaperones and helper proteins.

3.5. Hsp90 - A multitalented protein or simply a general chaperone?

At first glance, Hsp90's actions seem very contradictionary: *in vitro* Hsp90 presents itself as being a rather promiscuous chaperone, interacting with a broad range of folding and unfolding intermediates, whereas *in vivo* Hsp90 has been found associated only with a small subset of distinct substrate proteins. On closer examination, however, many similarities become apparent and the conclusion may be drawn that both the *in vitro* and *in vivo* functions of Hsp90 are based on the same mechanism: the transient and dynamic interaction of Hsp90 with non-native proteins.

All the known substrate proteins of Hsp90 seem to be in their non-native state yet possess a substantial amount of secondary and tertiary structure. This has been clearly demonstrated for thermally unfolding citrate synthase and is well established for kinases and steroid receptors. Hsp90 has been shown to interact with very early unfolding intermediates of citrate synthase. These intermediates are still in their dimeric state and have probably most of their native intramolecular contacts left. The same might also apply for casein kinase II, another *in vitro* substrate of Hsp90. Native casein kinase II precipitates probably due to partial unfolding and exposing "interactive" surfaces upon incubation in low salt buffer unless Hsp90 is present to bind the intermediates (53). The molecular basis of Hsp90's interaction with untransformed steroidreceptors has being discussed recently in a similar context as either i) regulating the oligomerisation state of receptors, ii) protecting receptors from proteolysis or iii) stabilizing their alternate conformational states (33). Taken together, a substantial amount of probably native like structure characterizes all known substrate proteins of Hsp90 and provides a link between the *in vivo* and *in vitro* substrate specificities of Hsp90.

Moreover, the mechanistical features of Hsp90 action with the different substrate proteins appear very similar. In either case, interactions with Hsp90 involve multiple rounds of binding, release and rebinding of the substrate protein. But then the question arises why no other proteins except steroidreceptors and kinases have been identified as *in vivo* substrates of Hsp90? The reason could be based on the nature of the two substrate proteins rather than on the substrate specificity of Hsp90. Steroid receptors and kinases represent polypeptides, where folding to the native state requires either specific ligands or association with the membrane, respectively. Highly structured, long-lived folding intermediates are therefore part of their folding pathway. In the absence of ligands or membranes, the equilibrium of folding is on the side of the folding intermediate and dissociation of Hsp90 provokes immediate rebinding. Hsp90 appears to be stably associated with the folding intermediates, keeps the polypeptides in a folding competent state and allows successful co-immunoprecipitation. Presence of ligands shifts the folding equilibrium towards the native state and rebinding to Hsp90 will not occur any longer. This folding pathway is in sharp contrast to the pathway of most other cellular proteins, which fold directly to the native state. This implies that the folding equilibrium is far shifted towards the native state and only transient associations with Hsp90 will be observed. The ATP independence of Hsp90's general chaperone activity fits nicely into this picture, since no major structural rearrangement are requested from this folding helper protein. The high abundance of Hsp90, however, is necessary for its action and guaranteed, since Hsp90 represents one of the most prominent proteins in the cytosol of eukaryotic cells.

4. ACKNOWLEDGMENTS

I thank Johannes Buchner and James Bardwell for many stimulating discussions and for critically reading the manuscript. I also thank David Smith for providing me with Figure 1. The work is supported by a fellowship of the Deutsche Forschungsgemeinschaft (DFG).

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