Chapter 4
Specification of Hsp70 Function by Type I and Type II Hsp40

Douglas M. Cyr and Carlos H. Ramos

Abstract Cellular homeostasis and stress survival requires maintenance of the proteome and suppression of proteotoxicity. Molecular chaperones promote cell survival through repair of misfolded proteins and cooperation with protein degradation machines to discard terminally damaged proteins. Hsp70 family members play an essential role in cellular protein metabolism by binding and releasing nonnative proteins to facilitate protein folding, refolding and degradation. Hsp40 family members are Hsp70 co-chaperones that determine the fate of Hsp70 clients by facilitating protein folding, assembly, and degradation. Hsp40s select substrates for Hsp70 via use of an intrinsic chaperone activity to bind non-native regions of proteins. During delivery of bound cargo Hsp40s employ a conserved J-domain to stimulate Hsp70 ATPase activity and thereby stabilize complexes between Hsp70 and nonnative proteins. Type I and Type II Hsp40s direct Hsp70 to preform multiple functions in protein homeostasis. This review describes the mechanisms by which Type I and Type II sub-types of Hsp40 bind and deliver substrates to Hsp70.

Keywords Hsp70 · Hsp40 · Protein folding · Molecular chaperone

Introduction

The Hsp40 family of co-chaperone proteins plays a role in cell stress protection, folding of nascent polypeptides, refolding of denatured or aggregated proteins, modulation of amyloid formation, protein degradation, and protein translocation. There are 44 *Hsp40* genes present in the human genome and 20 Hsp40s identified in the yeast genome (Kampinga and Craig 2010; Buchberger et al. 2010;

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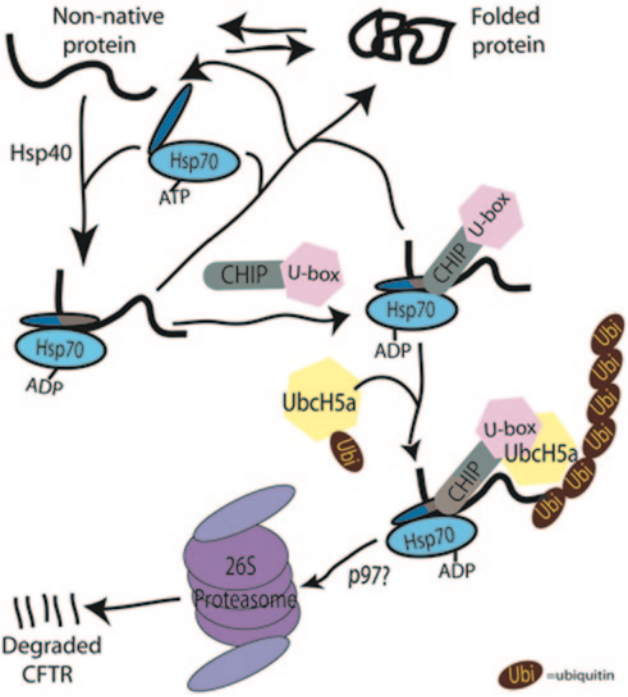

Fig. 4.1 Model for regulation of the Hsp70 polypeptide binding and release cycle by Hsp40. Hsp70 has low substrate affinity in the ATP bound state but upon hydrolysis of ATP stable Hsp-70substrate complexes are formed. Hsp70-substrate complexes then disassociate upon regeneration of Hsp70-ATP. In this model, Hsp40 acts to (1) deliver substrates to Hsp70 and (2) stimulate the ATPase activity of Hsp70. This cycle is repeated numerous times until the substrate protein is able to reach a native state. Co-chaperones such as the E3 ligase CHIP act downstream of Hsp40 to target Hsp70 clients to the proteasome for degradation

Kim et al. 2013; Cyr et al. 1994). These proteins were identified by the presence of a conserved J-domain that stimulates the ATPase activity of the Hsp70 (Fig. 4.1; Cyr et al. 1992; Liberek et al. 1991). Type I and Type II Hsp40s also have the conserved ability to bind and deliver non-native proteins to Hsp70, which is essential for life (Johnson and Craig 2001).

Type I Hsp40s are descendants of bacterial DnaJ and contain the J domain, followed by a glycine/phenylalanine rich region (G/F), a zinc finger like region (ZFLR), and a conserved C-terminal domain. The Type II Hsp40's are similar to the type I Hsp40s, but instead of the zinc finger like region they contain a glycine/methionine rich region. Type III Hsp40s contain the J-domain, but none of the other conserved domains found in Type I or II Hsp40s. Instead, they often have specialized domains that localize them to certain areas of the cell and provide specificity in substrate binding (Grove et al. 2011; Houck et al. 2014; Summers et al. 2013; Douglas et al. 2009). Type I and Type II Hsp40s contain a C-terminal dimerization

domain, but this does not mean that all Hsp40s function as dimers. However, the J-domains of Type III Hsp40s form dimers (Mokranjac et al. 2003), and the transmembrane Hsp40s DnaJB12 and DnaJB14, which lack a canonical dimerization domain, form heterodimers (Goodwin et al. 2014; Sopha et al. 2012). Thus, in many instances dimeric Hsp40s interact with Hsp70, but a general requirement for dimerization in Hsp40 function has not been demonstrated.

Hsp40s are conserved across species and are found in organisms from bacteria to humans, and a variety of Type I, Type II, and Type III Hsp40s are found in the same subcellular organelles where they can play specialized roles (Kampinga and Craig 2010). In order to better understand the cellular processes that these chaperones facilitate, we must first understand the mechanism by which Hsp40s bind substrates and regulate Hsp70 function. In the following sections, we will review the genetic, biochemical, cell biological, and structural data that have helped elucidate the unique mechanisms that different Hsp40s use to maintain protein homeostasis.

Hsp70 Co-Chaperone Activity of Hsp40s

The affinity of Hsp70 for polypeptides is regulated by its nucleotide bound state. In the ATP bound form, Hsp70 has a low affinity for substrate proteins. However, upon hydrolysis of the ATP to ADP, Hsp70 undergoes a conformational change that increases its affinity for substrate proteins (Fig. 4.1). Hsp70 goes through repeated cycles of ATP hydrolysis and nucleotide exchange, which permits cycles of substrate binding and release.

The Hsp70 proteins are assisted and regulated by several different co-chaperones. These co-chaperones have been shown to not only regulate different steps of the ATPase cycle of Hsp70 (Fig. 4.1), but they also have an individual specificity such that one co-chaperone may promote folding of a substrate while another may promote degradation. For example, the Hsp40 DnaJB12 and ubiquitin ligase CHIP both promote the degradation of Hsp70 bound substrates (Meacham et al. 2001; Cyr et al. 2002; Grove et al. 2011). On the other hand, the Hsp40 co-chaperones Hdj2 and Ydj1 promote protein folding (Meacham et al. 1999; Cyr and Douglas 1994; Fan et al. 2005a). The yeast Hsp40 Sis1 functions in spatial protein quality control (Douglas et al. 2008, 2009) and promotes protective aggregation of amyloid-like proteins (Wolfe et al. 2013, 2014). The Hsp40 proteins are classified as co-chaperones for Hsp70 due to the fact that they can use their various domain structures to (1) bind Hsp70 (2) help load the substrates on Hsp70 and (3) stimulate the ATPase activity of Hsp70 (Summers et al. 2009a; Cyr 2008). The general ability of Hsp40s to load substrates onto Hsp70 explains why Hsp40s are essential. The mechanism by which Hsp40s bind and interact with Hsp70s has been reviewed in detail, but many questions remain unanswered (Ramos et al. 2008; Sha et al. 2000; Fan et al. 2003, 2004, 2005a; Lee et al. 2002)..Therefore, in the remainder of this chapter we will focus on the question of how Hsp40s bind unfolded proteins.

Do Hsp40s Act as Chaperones?

It is established that Hsp40s specify Hsp70 function, but the manner by which Hsp40s bind and deliver substrates to Hsp40 is not completely understood (Fan et al. 2003; Summers et al. 2009a). Type I and Type II Hsp40s act independently as chaperones, so we will discuss the data that describes how they bind and transfer substrates to Hsp70.

The first observations of intrinsic chaperone activity of an Hsp40 came from studying the bacterial type I Hsp40, DnaJ (Langer et al. 1992) when purified DnaJ protein was shown to suppress the aggregation of denatured rhodanese. Subsequently, the yeast Hsp40 Ydj1 was shown to have the conserved ability to suppress protein aggregation (Cyr 1995; Lu and Cyr 1998a, b) and assist Hsp70 in refolding denatured proteins. These studies were the first to show that DnaJ and its eukaryotic relatives could not only bind denatured substrates, but it could also prevent the aggregation of those denatured substrates, thereby categorizing Type I Hsp40s as chaperones.

Studies with the yeast Sis1 protein, have shown that Type II Hsp40s can also bind chemically denatured luciferase and reduced α-lactalbumin and that this binding is dependent on specific residues within the C-terminal peptide-binding domain (Sha et al. 2000; Lee et al. 2002). This ability of Sis1 to recognize and bind non-native polypeptides classifies Sis1 as a chaperone. However, Sis1 alone is not as effective of a chaperone as the Type I Hsp40s because Sis1 cannot prevent the aggregation of thermally denatured luciferase nor does it hold the thermally denatured luciferase in a folding competent state. However, Sis1 is able to hold chemically denatured luciferase in a folding competent state (Lee et al. 2002) and also binds specific residues in yeast prions to promote prion propagation (Douglas et al. 2008). The human Hsp40, Hdj-1, also has the ability to bind non-native proteins and it's ability to recognize proline-rich regions of proteins (Lee et al. 2002) appears to make it susceptible to inactivation by huntingtin protein (Park et al. 2013). The inactivation of Sis1 by huntingtin is associated with inhibition of the proteasome and may contribute to huntingtin toxicity in the brain.

Determination of Functional Specificity

Type I and Type II Hsp40s bind exhibit different substrate specificity and direct Hsp70 to preform different functions *in vivo*. (Theodoraki and Caplan 2012; Fan et al. 2004; Rudiger et al. 2001; Caplan et al. 1992a, b, 1993; Luke et al. 1991). Sequence analysis reveals two possible regions that may be responsible for specifying this difference in function between the Type I and Type II Hsp40s. First the G/F rich region of Ydj1 and Sis1 are different, with that of Sis1 containing a 10 residue long insert containing the amino acids, GHAFSNEDAF (Yan and Craig 1999). Second, as mentioned previously, the protein modules located in the middle of Ydj1 and Sis1 are different such that Ydj1 contains the ZFLR and Sis1 contains the G/M region

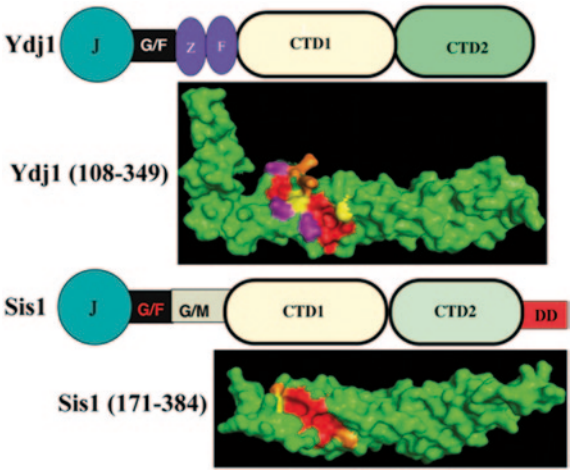

Fig. 4.2 Domain structures of Type I and Type II Hsp40 proteins. Ydj1 and Sis1 are yeast Hsp40s that are representative of Type I and Type II Hsp40 sub-types. The panels below the cartoons are models of Hsp40 substrate-binding domains built from X-ray structures of indicated Sis1 and Ydj1 fragments. *Red* denotes solvent exposed hydrophobic residues on the surface of the models. *J* J-domain, *G/F* glycine/phenylalanine rich region, *ZFLR* zinc finger-like region, *G/M* glycine/methionine rich region, *CTD1* carboxyl-terminal domain I, *CTD11* carboxyl terminal domain II, *DD* dimerization domain

(Fig. 4.2). Thus, it is plausible that either the G/F domain or the central domain (ZFLR vs G/M CTD1) of Ydj1 and Sis1 serve to specify their *in vivo* functions (Ramos et al. 2008; Fan et al. 2004). Below we will discuss the studies that were carried out in order to determine whether either of these differences has a role in specifying the functions of the Type I proteins versus the Type II proteins.

The G/F Region

To determine whether the G/F regions of Type I and Type II Hsp40s help specify Hsp70 functions the Craig group has carried out a number of complementation studies with Hsp40 fragments (Johnson and Craig 2001; Yan and Craig 1999). In these studies, which were conducted with a sis1Δ strain, the G/F region of Sis1, but not that of Ydj1, was shown to be important for suppression of lethality caused by the loss of Sis1 function. Deletion of the G/F region also prevents Sis1 from maintaining the prion state of RNQ1, while truncated versions of Sis1 containing just the J domain and G/F region (Sis1 1-121) can functionally substitute for wild type Sis1 (Aron et al. 2007). Deletion of one of the unique insertions of the Sis1 G/F region (Sis1 1-121 Δ101-113) causes a defect in cell growth in the absence of wild type Sis1, thereby suggesting that the unique insertion of the G/F region is at least partially responsible for specifying the *in vivo* functions of the Sis1 protein.

Sis1 ΔG/F still binds denatured luciferase and the RNQ1 protein, and Sis1 ΔG/F still stimulates the Hsp70 ATPase activity. The function that was lacking in the Sis1 ΔG/F protein was the ability to cooperate with Hsp70 to refold denatured substrates (Aron et al. 2005, 2007; Sondheimer et al. 2001; Johnson and Craig 2001). Sis1 ΔG/F can still bind substrates and stimulate ATPase activity, so the defect likely comes from an inability to efficiently transfer substrates from Sis1 to Hsp70. In support of this conclusion mutation of the conserved ASP-ILE-PHE (DIF) motif in the G/F region interferes with functions of Hsp40s that occur after J-domain dependent hydrolysis of ATP by Hsp70 (Cajo et al. 2006). Molecular details of G/F region action in Hsp40 function require further study (Wall et al. 1995), and this is an important topic because this domain clearly plays a critical role in regulation of Hsp70 function.

Central Domains

In addition to the differences found in the G/F regions, the central domains of the Type I and Type II Hsp40s also have dramatic structural differences(Borges et al. 2012; Silva et al. 2011; Ramos et al. 2008). The central domain of the Type II Hsp40s contains the G/M region and a polypeptide-binding site found in CTD1, while the Type I Hsp40s contain a ZFLR that is adjacent to CTDI. The differences in the substrate binding domains will be discussed in the next section, so for now we will concentrate on how the G/M region versus the ZFLR may help specify function. Studies with the full length Sis1 protein indicate that the G/M region has some overlapping function with the G/F region. As discussed above, deletion of unique residues within the G/F region has deleterious effects on cell growth in cells that only have a truncated version of Sis1 containing the J domain and G/F region (Aron et al. 2005; Johnson and Craig 2001). However, in cells expressing the full length Sis1, deletion of the same unique residues, Sis1 Δ101-113, no longer effects cell growth at normal temperatures. These cells also maintain the prion state of RNQ1. Likewise, deletion of the G/M region from the full-length protein (Sis 1 ΔG/M) has no effect on cell growth at normal temperatures and has a very mild effect on the maintenance of the RNQ1 prion. However, deletion of both the G/M and the unique residues within the G/F region from the full-length protein (Sis 1 ΔG/M Δ101-113) prevents the maintenance of the RNQ1 prion. These studies indicate that the essential function of Sis1 is actually specified by both the G/M region and the unique residues within the G/F region (Aron et al. 2005; Johnson and Craig 2001).

Studies of the ZFLR of Type I Hsp40s has also provided clues as to why the function of the Type I proteins is unique from the Type II proteins. While the central domain of the Type I Hsp40s, the ZFLR, has been implicated as a component of the polypeptide binding site in combination with CTDI. The exact role of the ZFLR is not completely clear. A NMR structure of the ZFLR reveals a V-shaped groove with an extended B-hairpin topology, which could potentially be involved in protein:protein interactions (Martinez-Yamout et al. 2000). A proteolytic fragment of Ydj1, Ydj1 (179–384), which is missing the J-domain and the first zinc binding

module of the ZFLR is capable of suppressing protein aggregation and therefore must retain the ability to bind substrates (Lu and Cyr 1998a, b). Therefore, while these studies do not rule out the possibility that the ZFLR is involved with polypeptide binding, it is definitely not required for polypeptide binding.

Mutation of the ZFLR does reveal that this domain is necessary to cooperate with Hsp70 in folding reactions (Fan et al. 2005b; Linke et al. 2003). In order to determine why the ZFLR is necessary to cooperate with Hsp70, yeast cells expressing a zinc-binding domain 2 (ZBD2) mutant of Ydj1 were examined. These cells show a decrease in the activity of the androgen receptor (AR), which is a known Hsp70 substrate. Isolation of androgen receptor complexes revealed that mutation of the ZFLR of Ydj1 leads to the accumulation of Hsp40-AR complexes with the concomitant decrease in Hsp70-AR complexes. Therefore, it seems that one important role of the ZFLR is to stimulate the transfer of substrates from Hsp40 to Hsp70 (Summers et al. 2009a).

In order to directly decipher the involvement of the ZFLR versus the G/M CTD1 central domains in specifying Hsp40 function, chimeric forms of Ydj1 and Sis1 were constructed in which the central domains were swapped to form YSY and SYS (Fan et al. 2004). Purified SYS and YSY were found to exhibit protein-folding activity and substrate specificity that mimicked that of Ydj1 and Sis1, respectively. *In vivo* studies also showed that YSY exhibited a gain of function, and unlike Ydj1, could complement the lethal phenotype of sis1Δ and promote the propagation of the yeast prion [RNQ1 +]. SYS exhibited a loss of function and was unable to maintain [RNQ1 +]. These *in vitro* and *in vivo* data suggest that the central domain of Ydj1 and Sis1 are exchangeable and that they help specify Hsp40's cellular functions.

Substrate Binding Domains

The studies discussed above suggest that the unique residues in the G/F region and the different central domains may help specify the function of the Type I vs Type II proteins by affecting the manner in which the individual chaperones interact with or transfer substrates to Hsp70. Another important determinant of specificity could obviously come from the substrate binding domains themselves. Since the Type I proteins do prefer to bind peptides that are distinct from those that the Type II proteins bind (Fan et al. 2004), one would hypothesize that there are differences in the substrate binding domains of these two types of proteins. Studies have shown that the substrate binding domains of both Type I and Type II Hsp40s are found in CTDI (Fig. 4.2; Sha et al. 2000; Qian et al. 2002). For example, the carboxyl terminus of the Ydj1 protein (residues 206-380) was shown to be at least partially responsible for polypeptide binding, and a single point mutation in this C-terminal domain (Ydj1 G315D) exhibits severe defects in cellular function and polypeptide binding (Lu and Cyr 1998a; Kimura et al. 1995). A fragment of Ydj1 consisting of residues 179-384 was also shown to be able to suppress rhodanese aggregation to the same level as the full length protein (Lu and Cyr 1998a). This fragment lacks the J domain, the G/F region and the first zinc-binding domain, but contains the

C-terminal domain. Studies of the yeast Type II Hsp40, Sis1, have also localized the polypeptide-binding site to CTDI (Sha et al. 2000; Lee et al. 2002). Therefore, similar regions within Ydj1 and Sis1 are implicated in polypeptide binding.

Crystal structures of the C-terminal domains of both Ydj1 and Sis1 have been solved (Fig. 4.2). These structures confirm that the C-terminal domain is a site for peptide binding for both types of Hsp40s and they suggest similar yet unique mechanisms for substrate binding. The Ydj1 crystal structure is of the monomeric form of a truncated C-terminal domain (Ydj1 102-350) in complex with a short peptide substrate, GWLYEIS (Li et al. 2003; Li and Sha 2003). There are two hydrophobic depressions, one in domain 1 and one in domain 3. The crystal structure shows that the peptide substrate binds to Ydj1 by forming an extra β-strand in the domain 1 depression. There is also an interaction in which the L from the peptide is buried in a small hydrophobic pocket found in this surface depression. The pocket that the L is buried in is formed by a variety of highly conserved hydrophobic residues (I116, L135, L137, L216, and P249), thereby suggesting that the pocket may be a common feature found in Type I Hsp40s, and may play a role in determining the substrate specificity.

The X-ray crystal structure of Sis1 171-352 was also solved and it depicts a homodimer that has a crystallographic two-fold axis (Sha et al. 2000; Qian et al. 2002). Sis1 171-352 monomers are elongated and constructed from two barrel-like domains that have similar folds and mostly β-structure. Sis1 dimerizes through a short C-terminal α-helical domain, and the dimer has a wishbone shape with a cleft that separates the arms of the two elongated monomers. CTDI on each monomer also contains two shallow depressions that are lined by highly conserved solvent exposed hydrophobic residues (Fig. 4.2). Mutational analysis of the residues that line the hydrophobic depression in Sis1 has identified K199, F201 and F251 as amino acids that are essential for cell viability and required for Sis1 to both bind denatured substrates and cooperate with Hsp70 to refold those substrates (Lee et al. 2002; Fan et al. 2004). Interestingly, peptides from the C-terminal lid domain of Hsp70 are also bound by in the hydrophobic depression on CTDI (Qian et al. 2002). It is therefore possible that Hsp70 and substrates interact with Sis1 at similar sites. If true, then Hsp70 might displace substrate from Hsp40s to drive substrate transfer from Hsp40 to Hsp70 polypeptide binding domain (Kota et al. 2009; Summers et al. 2009a).

Hsp40 Quaternary Structure

A common feature of Type I and Type II Hsp40s is that dimerization is important for them to function *in vivo* (Summers et al. 2009a, b). There are no crystal structures of full length Type I or Type II Hsp40s, but small angle X-ray scattering (SAXS) and protein modeling have been used to build models of the quaternary structure of Type I and Type II Hsp40s (Borges et al. 2005; Ramos et al. 2008). These models suggest that there are substantial differences in the quaternary structure of the Type

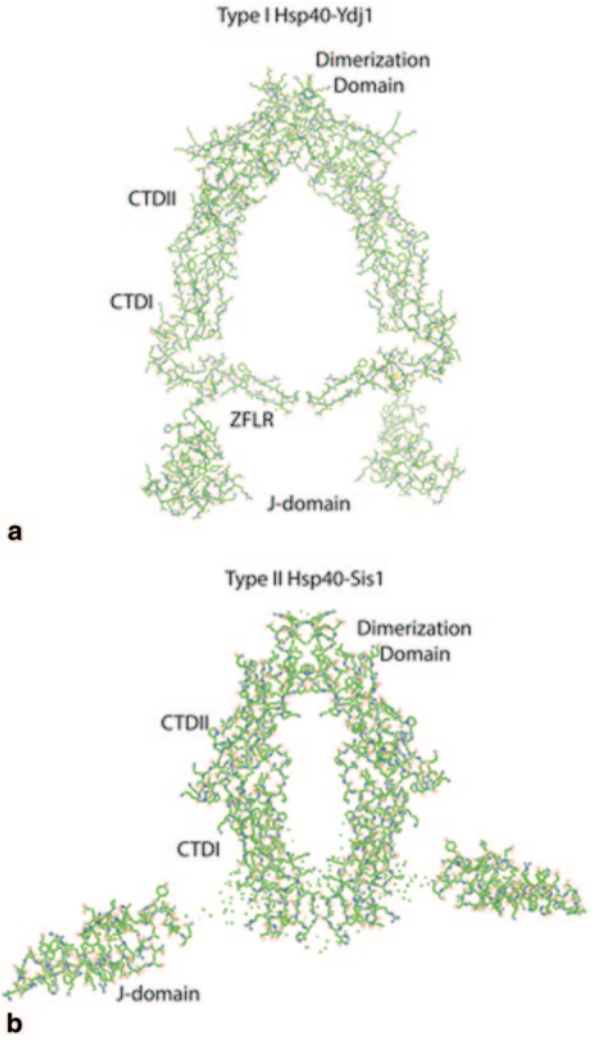

Fig. 4.3 Models of Type I and Type II quaternary structures. *Ab initio* models of Type I and Type II Hsp40s were generated by SAXS and molecular modeling of Ydj1 and Sis1. Models depicted were of Ydj1 and Sis1 dimers generated by pymol from data presented in Ramos et al. 2008

I and Type II Hsp40s that may help account for their ability to direct Hsp70 to preform different cellular functions (Fig. 4.3). In Type I Hsp40 the interface between CTDI and CTD2 and the ZFLR space the polypeptide binding pockets in CTDI and appear to impact the orientation for the J-domain relative to the long-axis of the chaperone (Silva et al. 2011). In Type II Hsp40s CDTI on the arms of the different dimers are closer together, and the J-domains are splayed to the side of the chaperone. It appears that J-domains can exist in a dimeric state, while the models depicted show the J-domains of Ydj1 and Sis1 as monomers. It is therefore possible that these models depict an inactive state of Ydj1 and Sis1 and that forms of these Hsp40s that regulate Hsp70 ATPase activity under go a conformational change to permit J-domain dimerization (Mokranjac et al. 2003; Goodwin et al. 2014).

A driving force for this putative conformational change might be the binding of polypeptides to the hydrophobic pocket in CTDI and downstream conformational changes. This model is hypothetical, and requires that an Hsp40 dimerize, and it is not clear that all Hsp40s are dimers. Never the less, this hypothesis suggests a substrate dependent mechanism for regulating some of the interactions that occur between Hsp40 and Hsp70.

While it appears that the unique structures of Type I and Type II Hsp40s almost certainly specify function, the exact mechanism by which these structures specify function is not clear. A combination of all the unique characteristics of the Type I and Type II chaperones discussed above likely explains the difference levels of chaperone and co-chaperone activity observed for these different types of Hsp40s.

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