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The M-Domain Controls Hsp104 Protein Remodeling Activity in an Hsp70/Hsp40-Dependent Manner

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Yeast Hsp104 is a ring-forming ATP-dependent protein disaggregase that, together with the cognate Hsp70 chaperone system, has the remarkable ability to rescue stress-damaged proteins from a previously aggregated state. Both upstream and downstream functions for the Hsp70 system have been reported, but it remains unclear how Hsp70/Hsp40 is coupled to Hsp104 protein remodeling activity.

Hsp104 is a multidomain protein that possesses an N-terminal domain, an M-domain, and two tandem AAA$^+$ domains. The M-domain forms an 85-Å long coiled coil and is a hallmark of the Hsp104 chaperone family. While the three-dimensional structure of Hsp104 has been determined, the function of the M-domain is unclear. Here, we demonstrate that the M-domain is essential for protein disaggregation, but dispensable for Hsp104 ATPase- and substrate-translocating activities. Remarkably, replacing the Hsp104 M-domain with that of bacterial ClpB, and vice versa, switches species specificity so that our chimeras now cooperate with the noncognate Hsp70/DnaK chaperone system. Our results demonstrate that the M-domain controls Hsp104 protein remodeling activities in an Hsp70/Hsp40-dependent manner, which is required to unleash Hsp104 protein disaggregating activity.

Keywords: molecular chaperone; AAA$^+$ machine; protein disaggregase; Hsp104; ClpB

Yeast Hsp104 is a ring-forming AAA$^+$ machine that, together with the cognate Hsp70 system (Hsp70/Hsp40), rescues proteins from a previously aggregated state.[1–3] In addition to its essential role in yeast stress response, Hsp104 is also required for the maintenance of all amyloid-based yeast prions[4] and for the elimination of [PSI$^+$], a yeast prion that increases translational read-through of nonsense codons.[5,6] The ability to remodel prions is not shared with the orthologous bacterial ClpB[6,7] and might be reflected in its three-dimensional structure.[8,9] However, we recently demonstrated that Hsp104 and ClpB share a remarkable structural conservation.[10–12] Our Hsp104 structure is consistent with a common mechanism for protein disaggregation and prion propagation,[13,14] and confirms the essential role of Hsp104 pore loops in substrate translocation.[15,16]

Hsp104 and ClpB are multidomain proteins consisting of an N-terminal domain (NTD), an M-domain, and two Walker-type ATPase domains (AAA$^+$).[10,12] The M-domain is a hallmark of the Hsp104/ClpB chaperone family and is inserted within the D1 small domain of the first AAA$^+$ (AAA-1) domain, similar to the β-domain of the distantly related Vps4 ATPase.[17] In solution, Hsp104 forms a homohexamer with the M-domains on the outside of the hexamer ring,[12] as they are in ClpB.[10,11] While the exact function of the

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Abbreviations used: NTD, N-terminal domain; T4L, T4 lysozyme; β-gal, β-galactosidase; FFL, firefly luciferase; MDH, malate dehydrogenase; FITC, fluorescein isothiocyanate.

Hsp104 M-domain remains unknown, its location on the Hsp104 exterior is consistent with a potential role in contacting large aggregated proteins and prion fibrils, as well as components of the bichaperone system.

It is widely accepted that the ability to solubilize protein aggregates requires a synergistic interaction between Hsp104 and the Hsp70 system in a species-specific manner.[18,19] Consistent with this notion, a direct interaction between bacterial ClpB and DnaK has been reported,[20,21] but its functional implication for protein disaggregation remains unclear. Since other Clp/Hsp100 proteins lacking an M-domain do not cooperate with the Hsp70/DnaK system in protein disaggregation,[22] it is conceivable that the M-domain might mediate the synergistic interaction between Hsp104 and Hsp70/Hsp40. To test this hypothesis, we used a biochemical approach to determine the functional role of the M-domain in chaperone activity. We demonstrate that the M-domain is essential for the solubilization of protein aggregates, but dispensable for the ATPase and protein translocating activities of unfolded substrates. Remarkably, we found that replacing the yeast Hsp104 M-domain with that of bacterial ClpB, and vice versa, switches the species specificity of the bichaperone system. Our results demonstrate that the M-domain controls Hsp104 function through direct interaction with the cognate Hsp70 system, which is required to unleash Hsp104 protein remodeling activity.

The Hsp104$_{T4L}$ chimera solubilizes a variety of heat-aggregated substrates

We previously reported the fitted electron cryomicroscopy structure of an engineered Hsp104 variant (Hsp104$_{T4L}$) that harbors T4 lysozyme (T4L) within the M-domain helix 2.[12] This Hsp104$_{T4L}$ variant is fully functional biochemically and, like wild-type Hsp104, cooperates with the Hsp70 system to reactivate heat-aggregated β-galactosidase (β-gal) and chemically denatured firefly luciferase (FFL).[12] Notably, Hsp104$_{T4L}$ has gained the ability to solubilize heat-aggregated β-gal in the absence of the Hsp70 system (Fig. 1a).[12] To determine whether Hsp104$_{T4L}$ can also recognize other aggregated proteins, we tested two additional heat-aggregated model substrates, namely, enhanced green fluorescent protein and malate dehydrogenase (MDH).

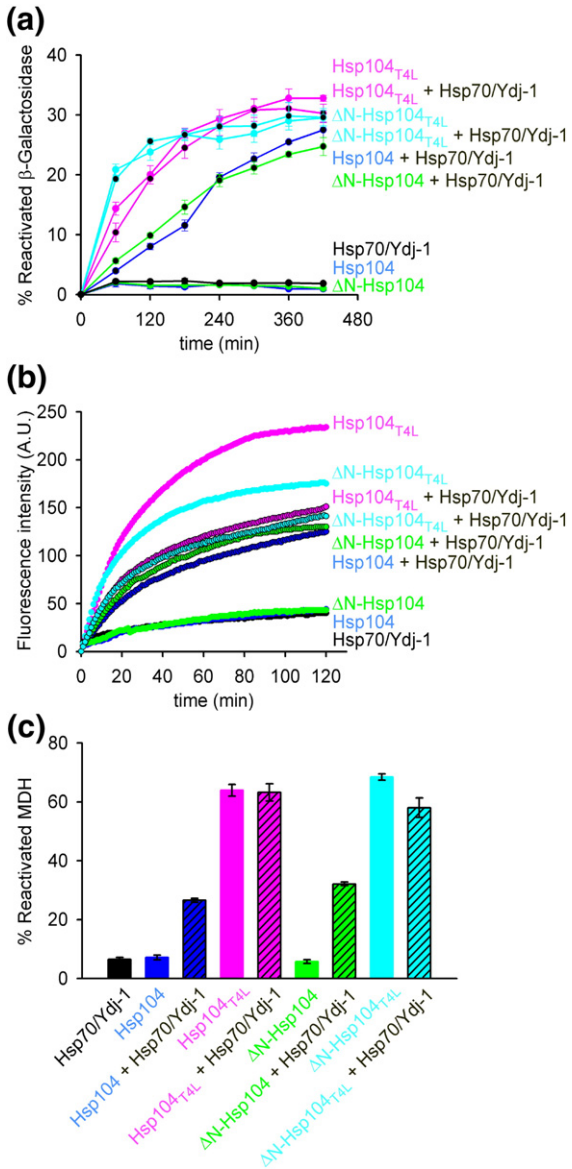

Fig. 1. Disaggregation of model substrates by Hsp104$_{T4L}$ fusion proteins. (a–c) Reactivation of protein aggregates by Hsp104, Hsp104$_{T4L}$, ΔN-Hsp104, or ΔN-Hsp104$_{T4L}$ (1 μM each) alone and together with Hsp70 and Ydj-1 (1 μM each). Assays contained an ATP regenerating system (20 mM phosphoenolpyruvate and 1 μM pyruvate kinase) and 4 mM ATP. Proteins were produced as described in Supplementary Material. (a) Reactivation of heat-aggregated β-gal was determined as described previously[12] and is shown over time as the percentage of native control. Standard errors of three independent assays are shown. (b) Enhanced green fluorescent protein (4.5 μM; BioVision, Mountain View, CA) was denatured at 80 °C in buffer A [25 mM Hepes (pH 7.5), 150 mM KOAc, 10 mM $Mg(OAc)_2$, and 10 mM DTT] for 10 min and diluted 1:10 with buffer A containing chaperones as indicated. The increase in fluorescence was measured with a Synergy HT microplate reader (BioTek, Winooski, VT) at 1-min intervals. A representative data set of three experiments is shown. (c) MDH (2 μM; Roche Diagnostics, Indianapolis, IN) was incubated in buffer A at 47 °C for 30 min and diluted 2.5-fold into buffer A at 22 °C with chaperones as indicated. MDH activity was determined after 180 min as described previously[23] and is shown as the percentage of native control. Standard errors of three independent assays are shown.

We found that $Hsp104_{T4L}$ can reactivate all three model substrates in the absence of the Hsp70 system (Fig. 1a–c). Notably, $Hsp104_{T4L}$ alone was able to reactivate heat-aggregated proteins perhaps even more efficiently than the wild-type bichaperone system (Fig. 1a–c). The higher efficiency of $Hsp104_{T4L}$ contrasts with previously reported activities[12] and is due to an improved protein purification procedure (details provided in Supplementary Material). No further enhancement in substrate reactivation was observed in the presence of the Hsp70 system, demonstrating that $Hsp104_{T4L}$ alone can solubilize a wide variety of heat-aggregated substrates.

The Hsp104 NTD is dispensable for protein disaggregation

Our $Hsp104_{T4L}$ variant provides the necessary means to investigate the Hsp104 protein remodeling activity free of other molecular chaperones and additional ATPases. Since the ATPase domains are conserved among AAA^+ machines, we reasoned that additional domains present in Hsp104 must confer the unique protein disaggregating function. While the Hsp104 NTD is dispensable for thermotolerance and luciferase refolding *in vivo*,[13,24] it is necessary for [PSI^+] prion inheritance[13] and curing by Hsp104 overexpression,[24] suggesting that the NTD confers some substrate specificity. To determine whether the Hsp104 NTD is important for protein disaggregation in the absence of the Hsp70 system, we engineered an $Hsp104_{T4L}$ variant lacking the NTD (ΔN-$Hsp104_{T4L}$). We found that ΔN-$Hsp104_{T4L}$ alone can solubilize all three heat-aggregated model substrates with similar efficiencies as $Hsp104_{T4L}$ (Fig. 1a–c). Our findings confirm that the Hsp104 NTD is dispensable for the disaggregation of heat-aggregated substrates, even in the absence of the Hsp70 system.

The Hsp104 M-domain is essential for protein disaggregation

To determine whether the Hsp104 M-domain is essential for protein disaggregation, we engineered an Hsp104 variant that lacks the M-domain in both wild-type background (Hsp104ΔM) and trap mutant background ($Hsp104\Delta M_{Trap}$) (Fig. 2a). We found that Hsp104ΔM forms a hexamer (Fig. 2b) and hydrolyzes ATP, which is stimulated in the presence of κ-casein (Fig. 2c). However, unlike wild-type Hsp104, Hsp104ΔM could not solubilize heat-aggregated model substrates in the presence of Hsp70/Hsp40 (Fig. 2d), indicating that Hsp104ΔM lost the ability to disaggregate amorphous aggregates. Notably, $Hsp104\Delta M_{Trap}$ still bound fluorescein isothiocyanate (FITC) conjugated casein, a soluble model substrate, with similar binding affinity as $Hsp104_{Trap}$ (Fig. 2e). Thus, our results demonstrate that the Hsp104 M-domain is essential for the disaggregation of heat-aggregated proteins, but is not required for binding of soluble model substrates.

Hsp104-ClpB chimeras are functional biochemically

To understand the function of the Hsp104 M-domain in protein disaggregation, we engineered Hsp104 chimeras that feature one or more domains of the orthologous *Escherichia coli* ClpB. It has previously been shown that Hsp104-ClpB chimeras can provide yeast thermotolerance *in vivo* as long as the AAA-1 domain of yeast Hsp104 is present,[13] suggesting that the AAA-2 domain is dispensable for Hsp104-specific chaperone activity.

The X-ray structure of ClpB showed that the AAA-1 domain is composed of a D1 large domain and a D1 small domain that features the M-domain inserted between helix C3 and β-strand c2.[10] To determine which segment of the AAA-1 domain may confer species specificity, we replaced the M-domain (Hsp104BM), the D1 small domain ($Hsp104BD_S$), or the entire AAA-1 domain of Hsp104 ($Hsp104BD_{LS}$) with the analogous domains of *E. coli* ClpB (Fig. 2a). All of our Hsp104-ClpB chimeras form hexamers, as determined by size-exclusion chromatography (Fig. 2b). We also observed additional shoulders and smaller peaks in chromatograms, indicating that our engineered chimeras are somewhat less stable than wild-type Hsp104 and Hsp104ΔM. However, our biochemical analysis showed that the ATP hydrolysis rates of $Hsp104BD_S$ and $Hsp104BD_{LS}$ are similar to those of wild-type Hsp104 (Fig. 2c), suggesting that the lower stability did not significantly affect the basal ATPase activities of those chimeras. Moreover, like wild-type Hsp104 and Hsp104ΔM, the ATPase activities of all three Hsp104-ClpB chimeras are further stimulated by κ-casein (Fig. 2c), albeit to a different extent. The complex nature of the basal and stimulated ATPase activities observed with our engineered chimeras is in line with the proposed role of the M-domain in coupling the ATPase activities of the AAA-1 and AAA-2 domains,[25] which is effected by the conformation and stability of the long coiled coil.[10,26–28]

Replacing the M-domain switches the species specificity of the bichaperone system

To identify the AAA-1 segment that confers the species specificity of the bichaperone system, we asked whether any of our Hsp104 chimeras could synergize with the bacterial DnaK system (DnaK/DnaJ/GrpE) to rescue aggregated proteins. To do so, we set up chaperone activity assays using

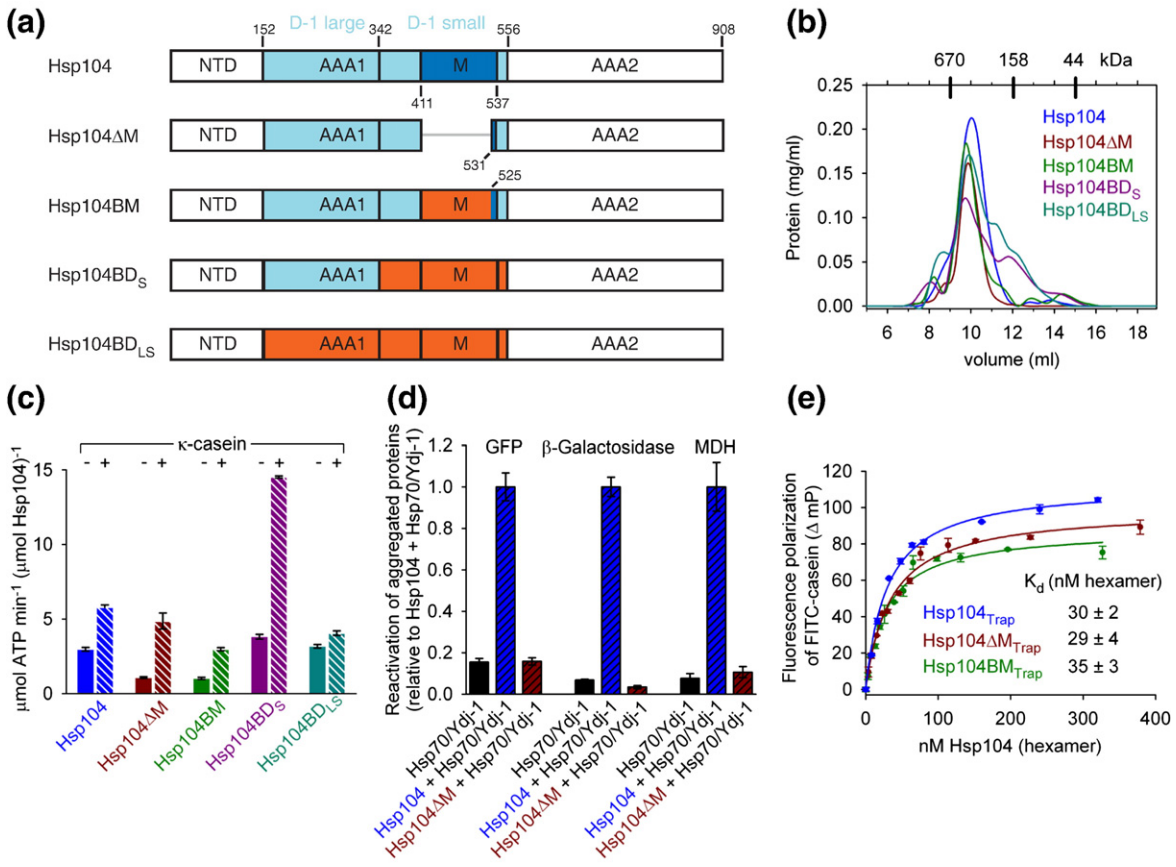

Fig. 2. Biochemical characterization of Hsp104 variants. (a) Domain arrangements of Hsp104, Hsp104ΔM, and Hsp104-ClpB chimeras. The AAA-1 domain is shown in blue, deletion of the M-domain is indicated by a gray line, and domains replaced by corresponding ClpB sequences are shown in orange. Hsp104ΔM and Hsp104-ClpB chimeras were constructed by cassette mutagenesis, expressed, and purified as described in Supplementary Material. (b) Hsp104, Hsp104ΔM, and Hsp104-ClpB chimeras (0.6 mg/ml) were incubated for 20 min at 22 °C in buffer A containing 2 mM ATPγS and 0.2 mg/ml κ-casein. Hexamer assembly was analyzed by size-exclusion chromatography on a Superdex 200 10/300 GL column (GE Healthcare, Piscataway, NJ) in buffer A plus 10 μM ATPγS. (c) The ATPase rates of Hsp104, Hsp104ΔM, and Hsp104-ClpB chimeras in the absence or in the presence of 0.2 mg/ml κ-casein were determined using a coupled ATP regenerating system at 30 °C in buffer A containing 2 mM ATP, as described previously.[12] Standard errors of three independent assays are shown. (d) Reactivation of aggregated model substrates by Hsp70 and Ydj-1 (1 μM each) alone and together with either Hsp104 (1 μM) or Hsp104ΔM (1 μM). Disaggregation assays were performed as described in Fig. 1a–c. Standard errors of three independent assays are shown. (e) Fluorescence polarization isotherms of the binding of FITC conjugated casein to Hsp104$_{Trap}$, Hsp104ΔM$_{Trap}$, and Hsp104BM$_{Trap}$. Equilibrium binding constants were determined at 22 °C by adding 0.15 μg/ml FITC conjugated casein (Sigma-Aldrich, St. Louis, MO) to Trap hexamers that were preassembled with 2 mM ATP in buffer A at 22 °C. Standard errors of three independent assays are shown.

either chemically denatured FFL (Fig. 3a) or heat-aggregated MDH (Fig. 3b) as substrate. Both aggregated FFL and MDH have been shown previously to function as model substrates for the yeast (Fig. 1c and Glover and Lindquist[18]) and the bacterial bichaperone system.[30,31]

Remarkably, all three Hsp104-ClpB chimeras cooperate synergistically with the DnaK system to solubilize aggregated model substrates, but lose their ability to function together with Hsp70/Hsp40 (Fig. 3a and b). Notably, wild-type Hsp104 cooperates only with Hsp70/Hsp40 and not with the bacterial DnaK system (Fig. 3a and b). Moreover, some substrate-specific differences were also observed. For instance, DnaK/DnaJ/GrpE can at least partially solubilize chemically denatured FFL, whereas the contributions of Hsp70/Hsp40 are negligible (Fig. 3a and c). It is conceivable that differences in substrate specificity, together with the reduced hexamer stability of our engineered chimeras (Fig. 2b), could explain the lower efficiency of the chimeric bichaperone system compared to native Hsp104/Hsp70/Hsp40 (Fig. 3a and b). Nevertheless, in our assays, the chimeric bichaperone system was always more efficient than the Hsp70 system or the DnaK system alone (Fig. 3a and b).

To confirm whether the M-domain is responsible for switching the species specificity of the bichaperone

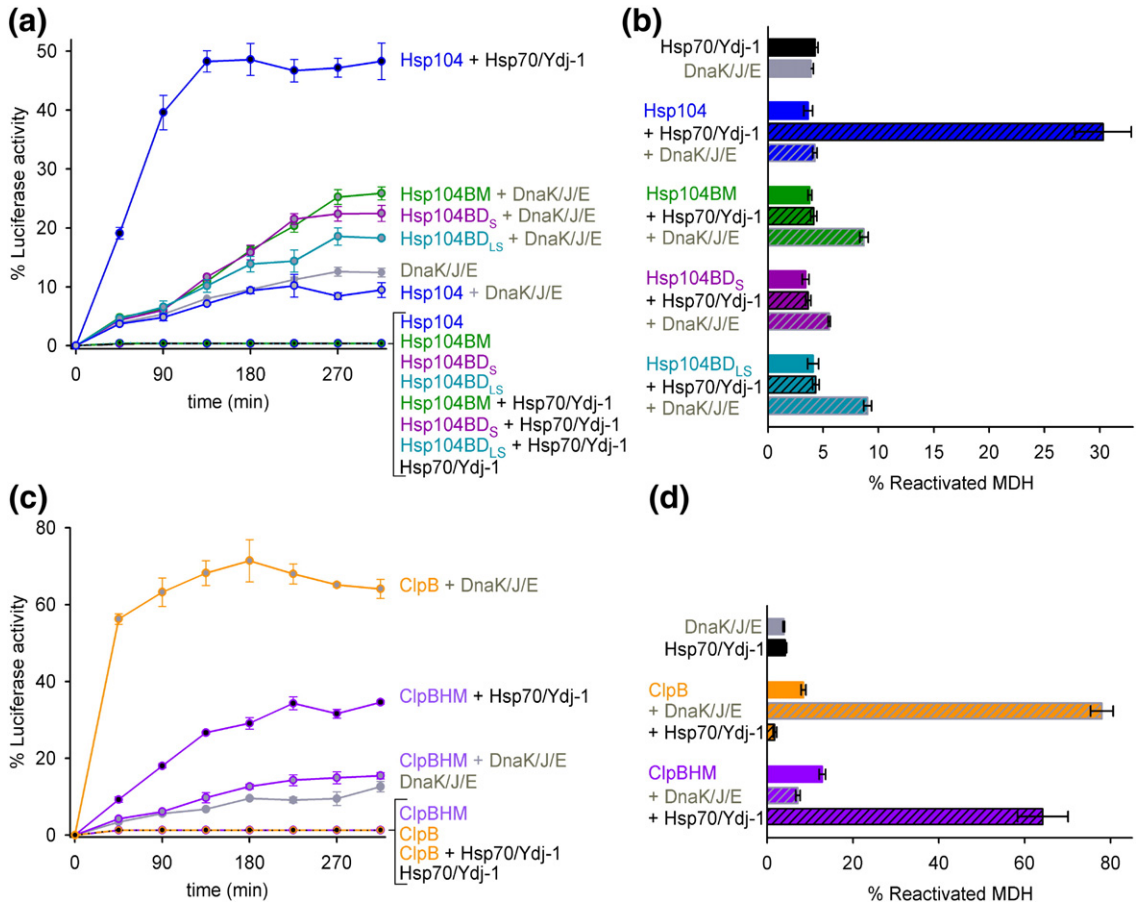

Fig. 3. M-domain replacement switches the species specificity of the bichaperone system. (a) Reactivation of chemically denatured FFL over time by Hsp104 or Hsp104-ClpB chimeras (1 μM each) alone and together with Hsp70 and Ydj-1 (1 μM each), or DnaK (0.86 μM), DnaJ (0.43 μM), and GrpE (0.25 μM). DnaK, DnaJ, and GrpE were purified as described in Supplementary Material. FFL (10 μM; Promega, Madison, WI) was denatured in 7 M urea, 25 mM Hepes (pH 7.5), 50 mM KCl, 15 mM $MgCl_2$, 10 mM DTT, and 5 mM ATP at 22 °C,[29] and diluted 125-fold into 25 mM Hepes (pH 7.5), 150 mM KCl, 15 mM $MgCl_2$, 2 mM DTT, and chaperones as indicated. Luminescence was measured at 22 °C, as described previously.[12] (b) Reactivation of heat-aggregated MDH by Hsp104 or Hsp104-ClpB chimeras (1 μM each) alone and together with Hsp70 and Ydj-1 (1 μM each), or DnaK (0.86 μM), DnaJ (0.43 μM), and GrpE (0.25 μM). Assay conditions were the same as in Fig. 1c. (c) Reactivation of chemically denatured FFL over time by ClpB or ClpBHM (1 μM each) alone and together with DnaK (0.86 μM), DnaJ (0.43 μM), and GrpE (0.25 μM), or Hsp70 and Ydj-1 (1 μM each). ClpB and ClpBHM were purified as described in Supplementary Material. Assay conditions were the same as in (a). (d) Reactivation of heat-aggregated MDH by ClpB or ClpBHM (1 μM each) alone and together with DnaK (0.86 μM), DnaJ (0.43 μM), and GrpE (0.25 μM), or Hsp70 and Ydj-1 (1 μM each). Assay conditions were the same as in Fig. 1c. Standard errors of three independent assays are shown in (a)–(d).

system, we asked whether the same is also true for ClpB. To address this issue, we replaced the ClpB M-domain with that of Hsp104 (ClpBHM) and analyzed the chimera using our chaperone activity assays. As anticipated, we found that ClpBHM cooperates synergistically with Hsp70/Hsp40, but not with DnaK/DnaJ/GrpE, whereas wild-type ClpB cooperates only with the bacterial DnaK system (Fig. 3c and d). While the efficiency of the reactivation of denatured FFL by ClpBHM/Hsp70/Hsp40 (Fig. 3c) was similar to that observed with the Hsp104 chimeras together with the DnaK system (Fig. 3a), reactivation of heat-aggregated MDH by ClpBHM/Hsp70/Hsp40 was nearly as efficient as the native bacterial bichaperone network (Fig. 3d). The latter is consistent with a different substrate preference for the yeast and the bacterial bichaperone system.

Taken together, our results demonstrate that the M-domain is essential for a synergistic interaction with the Hsp70/DnaK chaperone system and represents the minimal region required to switch the species specificity of the bichaperone network.

The M-domain couples Hsp70/Hsp40 to the Hsp104 protein remodeling activity

To determine whether the M-domain controls the ATP-driven Hsp104 motor activity required for substrate translocation through the Hsp104 hexamer, we generated Hsp104, $Hsp104_{Trap}$, Hsp104ΔM, and all of our Hsp104-ClpB chimeras as HAP variants by replacing Gly739, Ser740 and Lys741 with Ile-Leu-Phe. We found that all of our engineered HAP variants, including HAPΔM, can translocate and degrade FITC conjugated casein in the presence of ClpP (Fig. 4a). Neither ClpP alone nor HAP_{Trap} is able to do so, indicating that substrate translocation is driven by ATP hydrolysis, but does not require an M-domain or the presence of the Hsp70/Hsp40 system.

Unlike soluble substrates, it was previously shown that the degradation of protein aggregates by HAP/ClpP is dependent on Hsp70/Hsp40.[13,14] Consistently, we found that the HAP/ClpP-mediated degradation of heat-aggregated FAM-labeled MDH requires the M-domain and is strictly dependent on cooperation with the cognate Hsp70 system (Fig. 4b). Notably, we found that our Hsp104-ClpB chimeras alter the species specificity of the bichaperone network, so that our HAP chimeras now cooperate with the bacterial DnaK system, but no longer with eukaryotic Hsp70/Hsp40.

Together, our results demonstrate that the M-domain controls the Hsp104 protein disaggregating activity through functional interaction with the Hsp70 system in a species-specific manner.

Mechanistic model for protein disaggregation

Our results show that the Hsp104 M-domains are responsible for conferring the species specificity of the bichaperone network, presumably through direct interaction with the Hsp70 system. What might be the purpose of such an interaction? It has been proposed that Hsp104 recognizes substrates or is recruited to substrates in an Hsp70/Hsp40-dependent manner, followed by substrate translocation through the Hsp104 hexamer.[13,14,33] Alternatively, although not mutually exclusive, it has been proposed that the Hsp104 M-domains are required for the breakdown of large aggregates into smaller ones, followed by substrate translocation and handover to the Hsp70 system.[10,18] Consistent with both activities, we found that the M-domains are located on the Hsp104 exterior.[12] Notably, however, an Hsp70/Hsp40-dependent Hsp104–substrate interaction appears nonessential for protein disaggregation.[34,35] The latter is supported by our $Hsp104_{T4L}$ chimeras that can solubilize heat-aggregated substrates in the absence of the Hsp70 system (Fig. 1a–c).

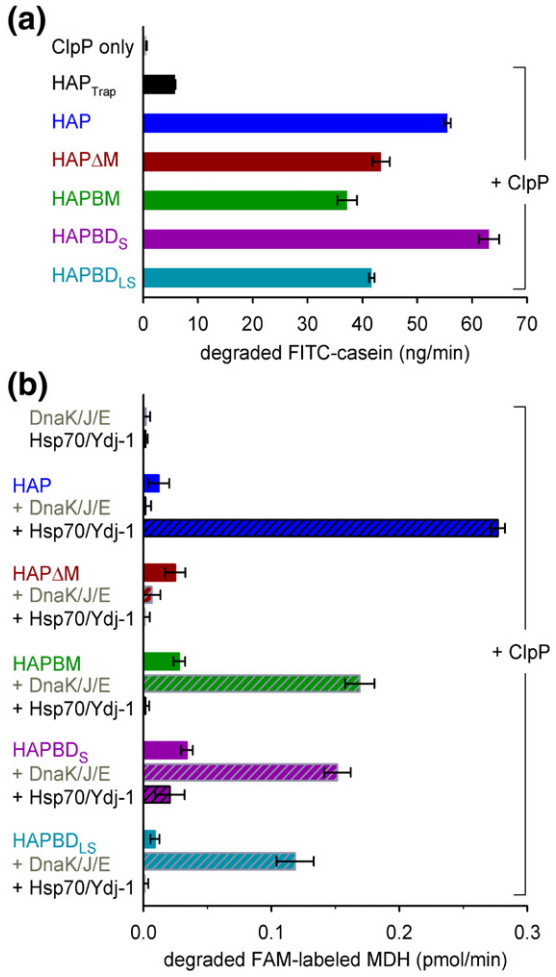

Fig. 4. Disaggregation requires M-domain-mediated collaboration with the Hsp70/DnaK system. (a) Degradation of FITC conjugated casein (0.12 mg/ml) by ClpP (2 μM) alone and together with HAP_{Trap}, HAP, HAPΔM, or HAP-ClpB chimeras (1 μM each). FITC conjugated casein was incubated in buffer A at 22 °C for 60 min with chaperones as indicated, together with an ATP regenerating system (6 mM phosphoenolpyruvate and 0.5 μM pyruvate kinase) and 3 mM ATP. Reactions were analyzed as described previously[32] by measuring the fluorescence signal of acid-soluble peptides using an LS55 fluorescence spectrometer (Perkin Elmer, Waltham, MA). Standard errors of three independent assays are shown. (b) Degradation of heat-aggregated FAM-labeled MDH (1.5 μM; labeling is described in Supplementary Material) by ClpP (2 μM) and HAP variants (1 μM) alone and together with DnaK (0.86 μM), DnaJ (0.43 μM), and GrpE (0.25 μM), or Hsp70 and Ydj-1 (1 μM each). Reactions were set up and analyzed in the same way as described for the FITC conjugated casein degradation assay (a). Standard errors of three independent assays are shown.

What might be the function of an Hsp40/Hsp70/Hsp104 M-domain interaction? We previously reported that the ability of $Hsp104_{T4L}$ to solubilize

heat-aggregated β-gal required the presence of the T4L moiety within the M-domain helix 2.[12] It is therefore tempting to speculate that the T4L moiety mimics a biologically relevant interaction, which is required for protein disaggregation. Consistent with our results obtained with the altered specificity chimeras, our findings point at a potential role for Hsp70/Hsp40 in activating the Hsp104 protein remodeling activity by binding to the M-domain. The latter is reminiscent of the known function of Vta1 in modulating Vps4 AAA^+ ATPase by binding to the β-domain that is inserted in the same place in Vps4[17] as the M-domain in Hsp104.

Together, our results provide an explanation for the upstream role of Hsp70/Hsp40 in Hsp104 function and support a regulatory mechanism common to diverse AAA^+ machines.

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Author Contributions. B.S. designed and performed research, analyzed data, and wrote the paper. F.T.F.T. designed research, analyzed data, and wrote the paper.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2010.07.030

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