Selective Hsp70-Dependent Docking of Hsp104 to Protein Aggregates Protects the Cell from the Toxicity of the Disaggregase

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Abstract

Hsp104 is a yeast chaperone that rescues misfolded proteins from aggregates associated with proteotoxic stress and aging. Hsp104 consists of N-terminal domain, regulatory M-domain and two ATPase domains, assembled into a spiral-shaped hexamer. Protein disaggregation involves polypeptide extraction from an aggregate and its translocation through the central channel. This process relies on Hsp104 cooperation with the Hsp70 chaperone, which also plays important role in regulation of the disaggregase. Although Hsp104 protein-unfolding activity enables cells to survive stress, when uncontrolled, it becomes toxic to the cell.

In this work, we investigated the significance of the interaction between Hsp70 and the M-domain of Hsp104 for functioning of the disaggregation system. We identified phenylalanine at position 508 in Hsp104 to be the key site of interaction with Hsp70. Disruption of this site makes Hsp104 unable to bind protein aggregates and to confer tolerance in yeast cells. The use of this Hsp104 variant demonstrates that Hsp70 allows successful initiation of disaggregation only as long as it is able to interact with the disaggregase. As reported previously, this interaction causes release of the M-domain-driven repression of Hsp104. Now we reveal that, apart from this allosteric effect, the interaction between the chaperone partners itself contributes to effective initiation of disaggregation and plays important role in cell protection against Hsp104-induced toxicity. Interaction with Hsp70 shifts Hsp104 substrate specificity from non-aggregated, disordered substrates toward protein aggregates. Accordingly, Hsp70-mediated sequestering of the Hsp104 unfoldase in aggregates makes it less toxic and more productive.

Introduction

Native proteins often possess a low margin of stability [1]. Changes in environmental conditions challenge conformation of these proteins and lead to their aggregation. This process is controlled and prevented by a group of proteins called molecular chaperones. Efficient disaggregation and refolding of proteins trapped in aggregates is crucial for thermotolerance in bacteria, plants, fungi and protozoans [2–4] and depends on the cooperation between the Hsp104/ClpB disaggregase (Hsp104 in yeast, ClpB in bacteria) and the Hsp70 chaperone [5–9]. Hsp104/ClpB disaggregases belong to AAA superfamily (ATPase Associated with various cellular Activities) and are built of three domains: the N-terminal domain and two nucleotide binding domains (NBD1 and NBD2) [10]. AAA unfoldases function as hexamers in which NBDs are spirally assembled, surrounding the central pore [9–12]. During disaggregation, polypeptides are extracted from the aggregate and translocated through this central pore in a ratchet-like manner, which is powered by ATP hydrolysis in NBDs [5,13–16]. The NBD1 domain contains an additional coiled coil region protruding from the hexamer, called the M-domain [10], which plays a regulatory role in Hsp104/ClpB disaggregases. It interacts with NBD1 and the neighboring M-domains in the hexamer in a head-to-tail way, forming a horizontal belt restricting conformational changes associated with ATP hydrolysis and protein

translocation, which keeps the disaggregase in a repressed, low activity state. Its repositioning causes disaggregase activation [17–20].

Hsp104/ClpB alone can bind and translocate disordered proteins, but for its key cellular function—protein disaggregation—it needs to collaborate with a cytoplasmic Hsp70 chaperone (Ssa1 in yeast, DnaK in bacteria), assisted by its co-chaperone Hsp40 [5,21]. Hsp70 regulates the disaggregase via the interaction with the M-domain, releasing Hsp104/ClpB from the repressed state, boosting its ATPase and protein-processing activities [18,22–27]. Our previous studies showed that the Hsp70-dependent activation of ClpB and Hsp104 can be reproduced by mutations that release the intramolecular interaction between the M-domain and NBD1 [28]. The resulting variants (e.g., Hsp104 D484K) are hyperactive: they display highly elevated activity, higher affinity for a protein substrate and are capable of disaggregation in the absence of Hsp70. These protein variants are highly toxic to the cell [29].

Due to their harmful potential, Hsp104/ClpB proteins require precise regulation to prevent unfolding of native proteins [28,30,31]. In our previous work [32], we showed that Hsp104 activity is limited not only by the M-domain-driven repression, but also by ADP inhibition, the latter being released only while a protein substrate is being translocated. At the same time, Hsp104 interaction with the protein substrate is determined by Hsp70, which supports Hsp104 in processing of aggregated substrates but not non-aggregated, disordered proteins [32]. Thus, Hsp70 activates Hsp104 specifically at the surface of an aggregate but not in the solution. These findings invited us for closer inspection of the regulatory role of cooperation with Hsp70 in disaggregation.

During initiation of disaggregation, a complex between Hsp70 and the disaggregase is formed. According to the model based on the NMR studies [26], in the bacterial bi-chaperone system ClpB–DnaK, the interaction site in *Tt*ClpB (*Thermus thermophilus* ClpB) is localized within the Motif-2 of the M-domain, with a crucial, conserved tyrosine on helix 3 at position 494. However, despite the fact that the structure and the basic mechanism of action are conserved in the bacterial and yeast bi-chaperone systems [33], the disaggregases apparently differ in the Hsp70-interaction site. The eukaryotic and prokaryotic chaperone partners are not interchangeable [5,21,22], and a mutation of the homologous tyrosine 507 in *Sc*Hsp104 does not abolish functional cooperation with the yeast Hsp70 [34]. A question of functional discrepancies between the individual systems has recently been addressed by several studies [29,33,35], yet the problem of the interaction site in the yeast system remains open.

Here, we investigate in detail the role and mechanism of Hsp70 involvement in Hsp104 binding to aggregated protein substrates. We develop a novel procedure based on Bio-Layer Interferometry (BLI) to monitor in real time complex formation between the disaggregase and Hsp70 at the surface of an aggregate. We also identify in the M-domain of Hsp104, a residue critical for interaction with Hsp70, and show that through this interaction, Hsp70 supports processing of aggregates over non-aggregated disordered proteins. Once the disaggregation initiation complex is formed, the same interaction results in allosteric activation of Hsp104. We demonstrate that these two mechanisms of Hsp70-dependent control are critical to avoid severe toxicity of the disaggregase.

Results

At the first stage of protein disaggregation, Hsp104 is delivered by Hsp70 to an aggregate and its potentially harmful protein-unfolding activity is released from repression. Given the importance of this initial phase for the processing of an appropriate substrate, lack of understanding of its mechanism is an issue. It remains unclear whether the crucial role of Hsp70 involves initial aggregate remodeling or rather a direct tethering of the disaggregase to the substrate. And if the latter is critical—does it involve the same mechanism as Hsp104 activation? To answer these and other questions concerning substrate recognition and binding by Hsp104, we set out to develop a real time method to investigate Hsp70-mediated docking of the disaggregase to protein aggregates.

Monitoring the Hsp104–aggregate interaction with BLI

We adopted BLI to monitor binding of chaperones to a model protein substrate: luciferase that had been heat-aggregated on the surface of the optical sensor. Contrary to SPR, BLI has an advantage of lacking a microfluidic system, which is prone to clogging during aggregate formation. When we incubated the aggregate-covered BLI sensor with either the yeast or bacterial Hsp70 system (Ssa1, Ydj1 or DnaK, DnaJ and GrpE, respectively), we detected an increase in a signal corresponding to the growing thickness of the bio-layer (Fig. 1a, Supplementary Fig. 1). In the presence of Ssa1 and Ydj1, when Hsp104 was added to the reaction, we observed its binding to the sensor, while ClpB did not bind (Fig. 1b). In the absence of Ssa1 and Ydj1, no interaction of Hsp104 with aggregates was detected (see Fig. 4b). In a reciprocal experiment, ClpB but not Hsp104 interacted with the sensor that had been incubated with the bacterial Hsp70 system (Fig. 1c). The observed decrease in the signal upon addition of ClpB can be attributed to the disaggregation reaction, which decreases the thickness of the protein layer on the sensor, and it shows that the bacterial disaggregase ClpB is more efficient in this experimental setup.

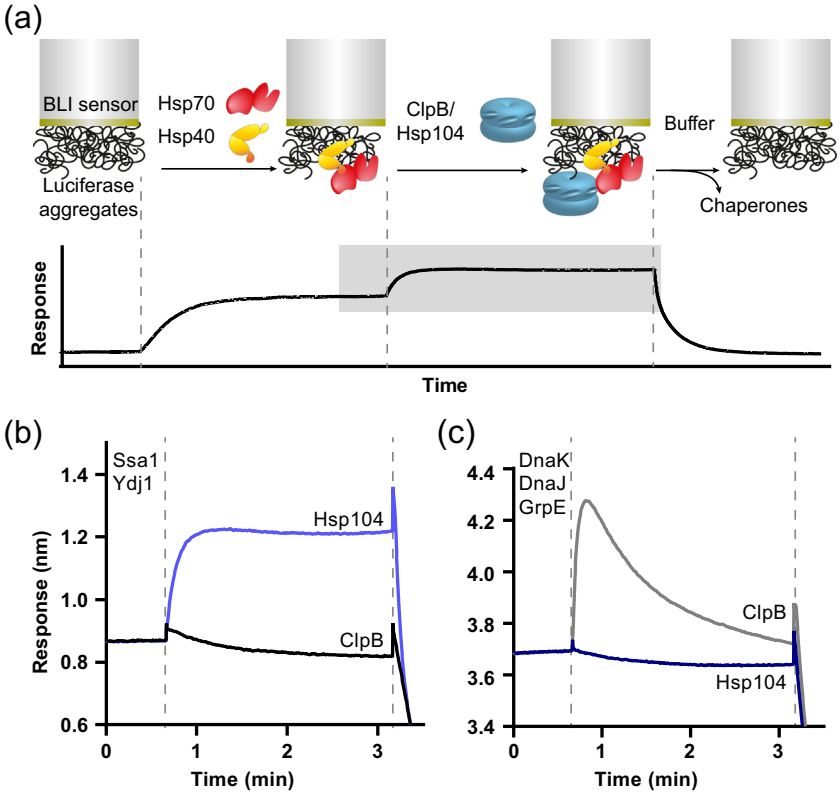


Fig. 1. Formation of the disaggregation initiation complex at the surface of an aggregate can be monitored with BLI. (a) Experimental scheme. BLI sensor covered with luciferase aggregates was incubated with the Hsp70 system, followed by the addition of the ClpB/Hsp104 disaggregase. (b) Hsp70 chaperone system from *S. cerevisiae* recruits Hsp104 but not orthologous *E. coli* ClpB to an aggregate. BLI sensor covered with luciferase aggregates was incubated in the presence of 5 mM ATP with Ssa1 and Ydj1 (1 and 0.8 μM, respectively) followed by addition of Hsp104 or ClpB (1 μM). (c) Bacterial Hsp70 chaperone system recruits ClpB but not Hsp104 to an aggregate. BLI sensor covered with luciferase aggregates was incubated with DnaK, DnaJ, GrpE (1, 0.4 and 0.3 μM, respectively) and 5 mM ATP, followed by addition Hsp104 or ClpB (1 μM).

These results correlate well with the previously reported observations that Hsp104 and ClpB cannot interact with aggregates without Hsp70 [36–38] and that the chaperone partners in the yeast and bacterial systems are not compatible [5,21–23]. This supports BLI as a suitable technique to investigate early stages of protein disaggregation.

Identification of the Hsp70-interaction site in Hsp104

To use BLI to discriminate between the initial events that depend solely on the Hsp70 activity and on the Hsp70–Hsp104 interaction, we needed to generate Hsp104 variant unable to bind Hsp70. In the case of the bacterial disaggregase ClpB, a critical tyrosine 494 has already been described as the interaction site and the Y494A mutation in *Tt*ClpB disrupted the interaction with DnaK [17,26]. However, it has been reported that a mutation in the homologous position in the yeast disaggregase did not produce analogous effects: stimulation of the Hsp104 Y507A mutant by Ssa1 was still observed [34]. These results, as well as the incompatibility between the systems [5,21–23], gave the assumption of fundamental differences in the mechanism of collaboration with Hsp70 in the yeast and bacterial disaggregases [29,35].

To solve this controversy, we compared the amino acid sequences of disaggregases from 28 species representing distinct groups of organisms (Fig. 2). We arranged the aligned sequences according to the relation between the organisms [39,40]. Inspection of the M-domain region that contains the aromatic residue involved in the interaction with Hsp70 in bacteria (the universally conserved tyrosine or phenylalanine, homologous to Y507 from *Sc*Hsp104) shows that there is an additional aromatic residue (tyrosine or phenylalanine) in the position +1 in all of the disaggregases from fungi and other eukaryotes from the Unikonta/Amorphea supergroup (Fig. 2). The additional aromatic residue is not present in the

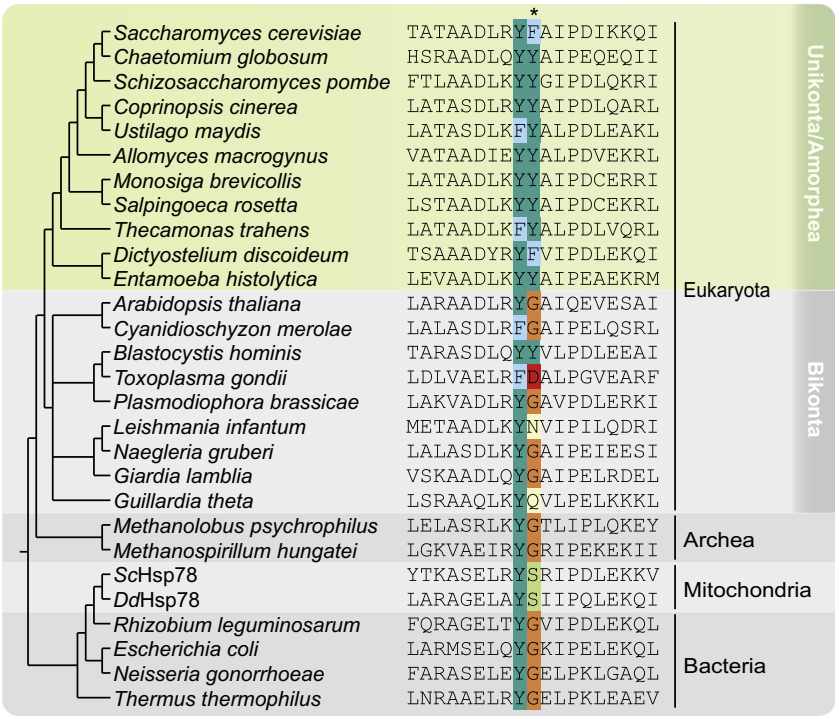


Fig. 2. Sequence alignment of the M-domain fragment from various disaggregases. Alignment of sequences homologous to the fragment T499–I517 from *Sc*Hsp104 protein. Residues homologous to Y507 and F508 (column indicated with an asterisk) are highlighted according to the chemical character of the residue: Y, cyan; F, light blue; G, orange; D, red; N and Q, yellow; S, green. The sequences have been arranged according to the relations among organisms, indicated with the cladogram on the left, constructed according to [39,40]. Due to the endosymbiotic origin of mitochondria, mitochondrial Hsp78 proteins have been grouped together with ClpB from Alphaproteobacteria. The Unikonts/Amorphea supergroup is indicated with green background.

mitochondrial disaggregases and in the proteins from Bacteria, Archea, and the Bikonta supergroup of Eukaryotes.

We assumed that the aromatic residues in the analyzed motif of the M-domain are critical for the interaction with Hsp70. The fact that Hsp104 has two such residues could explain why the Y507A mutation alone does not impair collaboration with Hsp70, whereas homologous substitution in the bacterial system does, as it affects the only aromatic residue in the region. To verify this hypothesis, we generated a double mutant Hsp104 Y507A F508A and tested it for cooperation with Hsp70 in disaggregation of heat-aggregated luciferase. As expected, the activity of luciferase was efficiently recovered by Hsp104 WT and the Y507A variant in the presence of Ssa1 and Ydj1, but the double mutant Y507A F508A was only marginally active (Fig. 3a). The disaggregation activity of Hsp104 Y507A F508A with Hsp70 was similar to the sum of activities measured for the chaperone partners separately, showing that this variant of the disaggregase is not stimulated by Ssa1 (Fig. 3b). We obtained analogous results with aggregated green fluorescent protein (GFP) as a protein substrate (Fig. 3a,b). To establish the minimal change sufficient for the observed effects, we performed the same experiments using Hsp104 with a single-substitution, F508A. This variant did not collaborate with Ssa1 either (Fig. 3a,b), which shows that in yeast disaggregase it is phenylalanine at position 508, not tyrosine 507, that is critical for functional cooperation with Hsp70. Thus, the interaction site in both yeast and bacterial disaggregases involves an aromatic residue located in the Motif 2 of the M-domain, although its exact position is not globally conserved.

It is noteworthy that all the analyzed Hsp104 variants: Y507A, Y507A F508A and F508A, in contrast to the WT, are capable of disaggregation without the aid of Hsp70, albeit the activity is low due to inhibition by the accumulating ADP (Supplementary Fig. 2a). When we added an ATP regeneration system, aggregated GFP was reactivated with high efficiency (Supplementary Fig. 2b), all of which is typical for hyperactive Hsp104. Moreover, the activity of Y507A and F508A measured without Hsp70 was similar (Supplementary Fig. 2a,b), which indicates that the F508A substitution decreases the disaggregation activity in the presence of Hsp70 not due to the protein being non-functional but because of disrupted interaction with the chaperone partner.

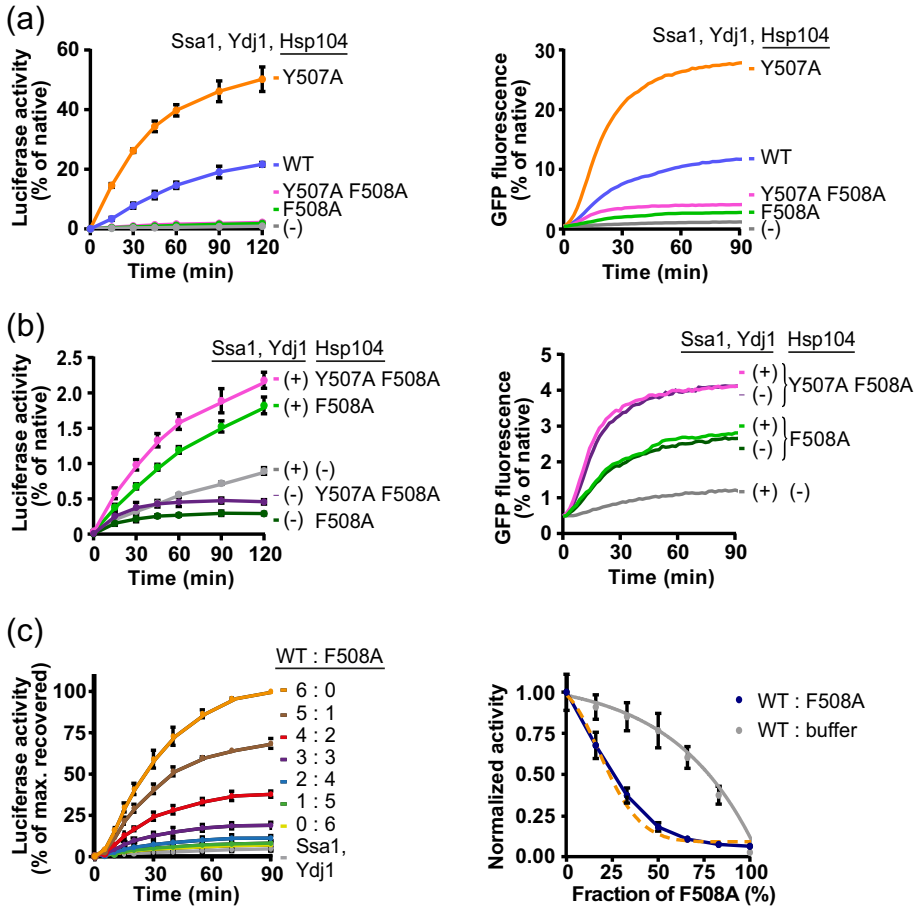


Fig. 3. Phenylalanine 508 in Hsp104 M-domain is responsible for functional cooperation with Ssa1 in disaggregation and refolding. (a) Refolding of urea denatured luciferase (left panel) by either Hsp104 WT, Hsp104 Y507A, Hsp104 Y507A F508A or Hsp104 F508A (0.5 μM) in the presence of Ssa1 (2 μM) and Ydj1 (1 μM). Refolding of heat-aggregated GFP (right panel) by either Hsp104 WT, Hsp104 Y507A, Hsp104 Y507A F508A or Hsp104 F508A (1 μM) in the presence of Ssa1 (1 μM) and Ydj1 (1 μM). (b) Refolding of urea denatured luciferase (left panel) or heat-aggregated GFP (right panel) performed by Hsp104 Y507A F508A or Hsp104 F508A in the presence or absence of Ssa1 and Ydj1. Protein concentrations as in panel a. (c) Hsp104 is sensitive to the incorporation of Hsp70 binding-deficient subunits. Refolding of urea denatured luciferase (left panel) performed with the different mixtures of Hsp104 and Hsp104 F508A in the presence of Ssa1 (2 μM) and Ydj1 (1 μM). The total concentration of Hsp104 variants is 0.5 μM. Mixing ratios are indicated (right panel, blue curve). Normalized initial luciferase refolding rates calculated from the curves presented in the left panel. As a reference, the normalized initial refolding rates (gray curve) are shown for WT Hsp104 diluted with buffer instead of Hsp104 F508A (WT: buffer). Yellow dashed line presents a theoretical activity plot made assuming that only the wild-type homohexamers within the mixture of assemblies remain active. The theoretical activity for each molar ratio of Hsp104 variants has been calculated with the assumption that the two Hsp104 variants form various hexamer species according to binominal probability distribution (Supplementary Fig. 3) and corrected for the observed non-linear effect of Hsp104 WT dilution. Data for luciferase are the mean (±SD) of three independent experiments.

Hsp104 hexamers show rapid exchange of subunits [41]. When the wild-type and the mutant protein variants are mixed, they form a combination of heterohexamers (Supplementary Fig. 3a). Compositions of such mixtures can be calculated for various protein ratios using the binominal distribution (Supplementary Fig. 3b) [35,42]. Hexamer tolerance for mutant subunits can be inferred from a comparison between the theoretical activities derived from a model and the experimental data [35,42]. To determine the minimal number of the F508A subunits sufficient to abolish collaboration with Hsp70, we mixed Hsp104 wild type with the F508A mutant in various proportions and tested for reactivation of heat-aggregated luciferase (Fig. 3c). In a control experiment, we established the effect of dilution on the activity of the wild-type protein (Fig. 3c). Incorporation of the mutant subunits resulted in a sharp decline in the disaggregation activity (Fig. 3c). The assumption that only the population of wild-type homohexamers within the mixture of assemblies remains active leads to a theoretical activity plot, which closely matches the

results of the luciferase disaggregation assay (Fig. 3c). This suggests that incorporation of even one Hsp104 F508A subunit cripples the hexamer's cooperation with Hsp70.

Next, to examine the importance of the interaction with Hsp70 in aggregate recognition and binding, we employed the Hsp104 F508A variant in the BLI experiment. As previously, we incubated the luciferase aggregates immobilized on the sensor with the yeast Hsp70 system, and upon addition of WT Hsp104 as well as the Y507A variant, we detected binding (Fig. 4a). In comparison, for the F508A variant, we observed only a slight increase in the signal (by 0.1 nm) (Fig. 4a), which indicates that substrate binding by the disaggregase strongly depends on the interaction with Hsp70. When we incubated the aggregate-covered sensor with Hsp104 in the absence of the Hsp70 system, the F508A and Y507A mutants, but not the wild type Hsp104, were capable of substrate binding (Fig. 4b), which correlates well with the Hsp70-independent disaggregation activity of the hyperactive variants (Supplementary Fig. 2a, b). This shows that the interaction with the aggregate observed for F508A in the presence of Hsp70 can be attributed rather to its hyperactivity than to the Hsp70 assistance. In fact, Hsp104 F508A binding signal was lower in the presence of Hsp70 than in its absence (Fig. 4a, b), which points to competition rather than cooperation between Hsp104 and Hsp70. These results suggest that the critical role of Hsp70 at the initial phase of disaggregation involves the physical docking of Hsp104 to the substrate rather than Hsp104-independent rearrangements of the aggregates.

The latter mechanism of stimulation by Hsp70 upstream of the disaggregase has been proposed based on the observation that incubation with the bacterial Hsp70 system (DnaK, DnaJ and GrpE) affects sedimentation of GFP aggregates in glycerol gradient [43]. We performed an analogous experiment using the yeast Hsp70 system. Incubation with Ssa1 and Ydj1 resulted in significantly slower sedimentation of aggregated GFP (Supplementary Fig. 4a). To assess if such remodeling of the aggregates stimulates disaggregation by the F508A variant, we incubated aggregated GFP in the presence or absence of the Hsp70 system for 1 h and used it as a substrate in the disaggregation reaction (Supplementary Fig. 4b). To avoid inhibition of Hsp104 by ADP produced by Ssa1 during the pre-incubation, we used an ATP-regeneration system, which boosts the Hsp70-independent activity of the hyperactive Hsp104 (Fig. 6, Supplementary Figs. 3 and 4b) [28]. In the case of F508A, the use of the Hsp70-remodeled aggregates in comparison to the standard aggregates increased the disaggregation rate by 80% (Supplementary Fig. 4b). This stimulation can be attributed to Hsp104-independent Hsp70 activity, as without the pre-incubation, Hsp70 does not support disaggregation by F508A (Fig. 3b, Supplementary Fig. 4b). A question arises, what is the physiological relevance of remodeling of aggregates by Hsp70 in comparison to the Hsp104–Hsp70 interaction? We addressed this question with phenotype analysis of the *hsp104 F508A* mutant.

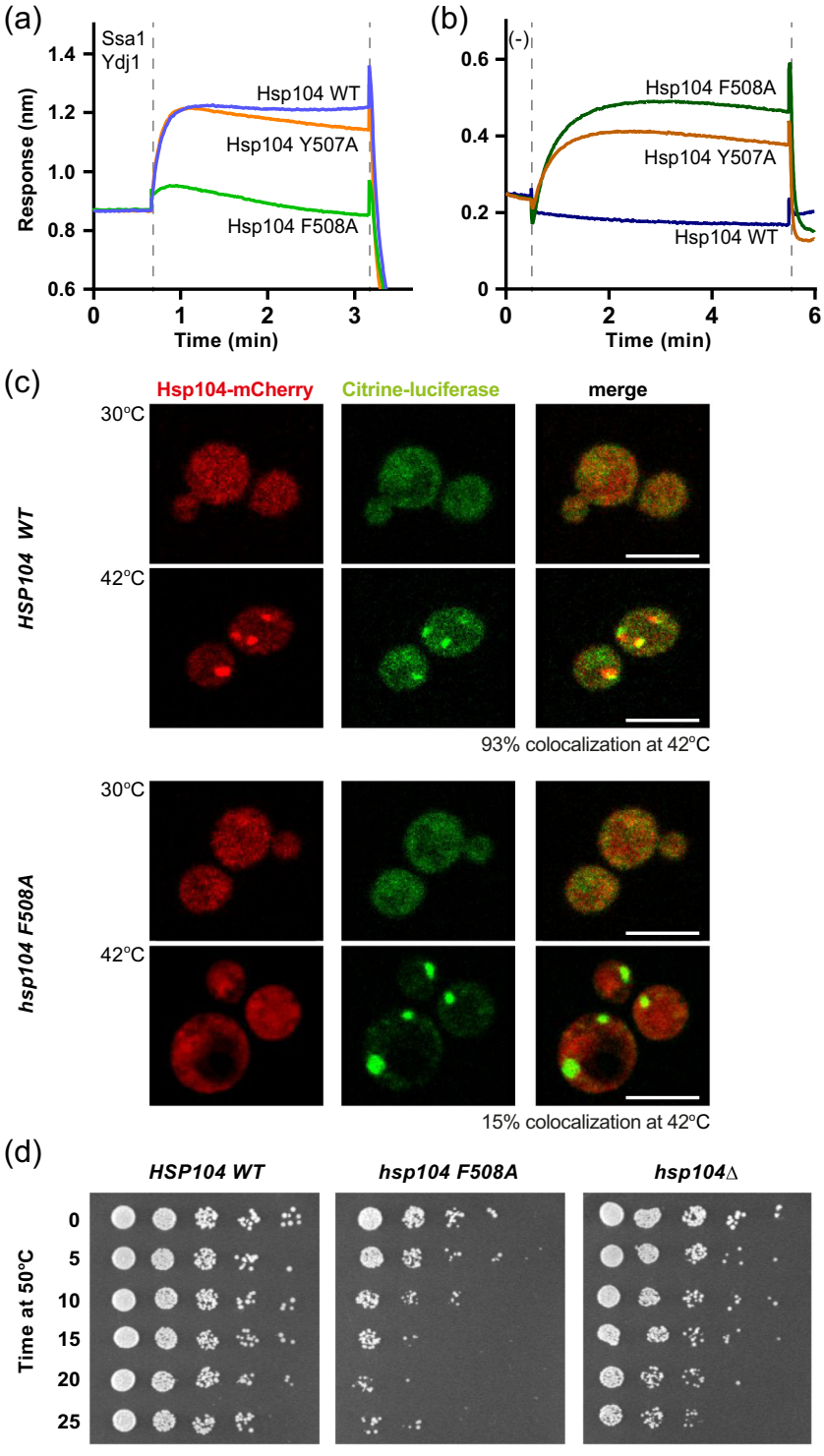
In vivo effects of disruption of the Hsp70-binding site in Hsp104

Since in yeast cells subjected to heat stress Hsp104 localizes to aggregates [36], we tested the impact of the F508A mutation on the cellular localization of the disaggregase. We fused *HSP104* and *hsp104 F508A* genes with mCherry, and used a yeast strain expressing thermolabile luciferase fused with citrine, which allows to visualize aggregates during heat stress. As reported previously [36], fluorescence from Hsp104–mCherry and non-aggregated citrine–luciferase was dispersed throughout the cytosol at 30 °C (Fig. 4c). Once the cells have been transferred to 42 °C, citrine–luciferase and Hsp104–mCherry formed colocalizing fluorescent foci (Fig. 4c). In yeast carrying the *hsp104 F508A*–mCherry gene fusion, the fluorescence corresponding to the disaggregase remained dispersed at 30 °C and was observed only in 15% of foci formed by citrine–luciferase at 42 °C (Fig. 4c). To be sure that the F508A variant is not only defective in interaction with luciferase aggregates and that the observed effect is not limited to the Hsp104 fusion with mCherry, we used analogous chromosomal fusions of *HSP104* and *hsp104 F508A* with *GFP* and analyzed if the resulting fluorescent disaggregases would localize to the protein aggregates formed at the heat shock conditions. As reported previously [44], in 99% of the cells expressing *HSP104–GFP*, fluorescent foci were observed at 42 °C (Supplementary Fig. 5). In contrast, in the cells carrying the F508A mutation, fluorescence

Fig. 4. Effective binding of Hsp104 to the aggregates depends on its interaction with Hsp70. (a) Effect of the interaction with Hsp70 on Hsp104 recruitment to luciferase aggregates. BLI sensor covered with luciferase aggregates was incubated either with Ssa1, Ydj1 (1 and 0.8 μM, respectively) and 5 mM ATP (a) or without the Hsp70 system (b), followed by addition of either Hsp104 WT, Hsp104 Y507A or Hsp104 F508A (1 μM) in the presence of 5 mM ATP. (c) Co-localization of Hsp104 WT or Hsp104 F508A with heat-induced luciferase aggregates in yeast cells *S. cerevisiae* cells with genome-borne *HSP104–mCherry* or *HSP104 F508A–mCherry* and *citrine–luciferase* were grown at 30 °C and transferred to 42 °C for 10 min to induce luciferase aggregation. The scale bars represent 5 μm. (d) Hsp104 F508A does not provide thermotolerance. An equal number of *S. cerevisiae* W303 *hsp104*Δ, W303 *HSP104* or W303 *HSP104 F508A* cells were transferred to 37 °C to induce the heat shock response. Subsequently, the cells were transferred to 50 °C. At the indicated time points, cells were serially diluted, spotted onto SC (-His) plate and grown for 2 days at 30 °C.

remained dispersed and only in 16% of the cells fluorescent foci were present (Supplementary Fig. 5). Thus, both the localization experiments show that the F508A variant possesses very low potential of interacting with aggregates at the heat shock conditions.

We also analyzed the ability of *hsp104 F508A* to confer tolerance to heat. We transferred yeast cells from a 15-min pre-shock at 37 °C to 50 °C and examined cell survival over time (Fig. 4d). The WT Hsp104 and Hsp104 F508A levels upon expression from a centromeric pRS313 plasmid under native promoter were similar, as verified with Western blot (Supplementary Fig. 6). In contrast to the wild type, the *hsp104 F508A* cells were susceptible to the heat stress



even more than the deletion strain, implying that the mutant does not confer thermotolerance (Fig. 4d). Similarly, no thermotolerance was observed for the chromosomal *hsp104 F508A–gfp* fusion used in the localization studies (Supplementary Fig. 5b). All the above *in vivo* results show that the F508-dependent interaction with Hsp70 is indispensable for Hsp104 association with aggregates during heat shock and, in consequence, for its critical cell-protecting function during stress.

The decreased survival upon expression of the *hsp104 F508A* mutant (Fig. 4d) could result from the protein's hyperactivity, manifested in the Hsp70-independent disaggregation activity (Supplementary Fig. 2a,b) and aggregate binding (Fig. 4b). We compared the effects of F508A with the previously described D484K mutation, which introduces repulsion between the M-domain and NBD1 and induces strong hyperactivity. When expressed from the centromeric plasmid in the Δ*hsp104* yeast strain, *hsp104 F508A* was only slightly toxic in comparison to the *D484K* mutant, which strongly impaired cell growth (Fig. 5a). The harmful effect correlated with the activity measured without the Hsp70 system: Hsp104 D484K alone bound to aggregates immobilized on the BLI sensor and reactivated aggregated luciferase and GFP more effectively than Hsp104 F508A (Fig. 5b, d). The ATPase activity of D484K comparing to the F508A variant was also higher (Supplementary Fig. 7a). This suggests that the F508A substitution disturbs the M-domain and makes Hsp104 hyperactive, but not to a full extent.

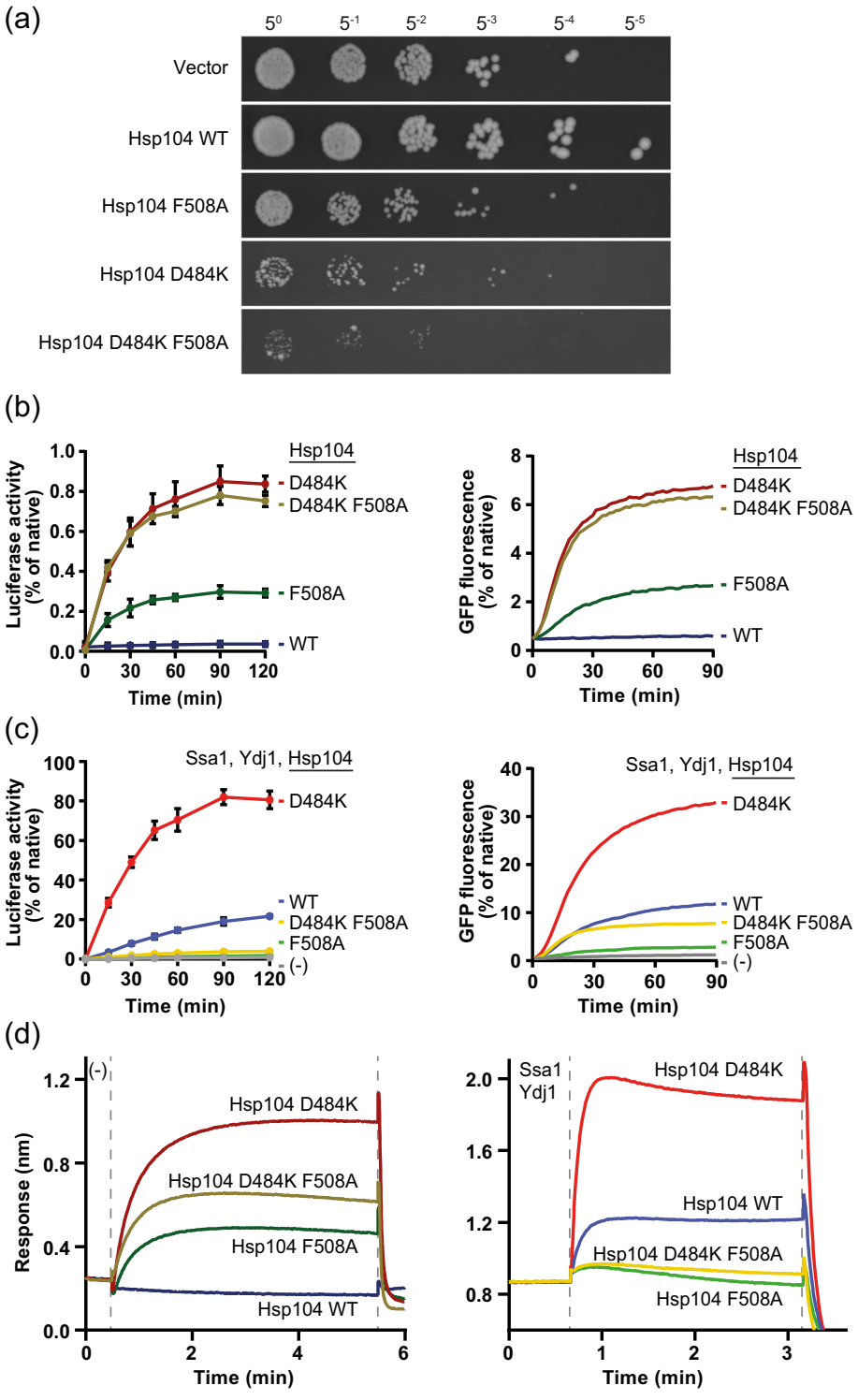
Considering that Hsp104 D484K alone remains fully or nearly fully activated independently of Hsp70, we asked what impact on the cell it would have if we additionally disabled its ability to interact with the chaperone partner. We generated a double mutant *D484K F508A*. Interestingly, when expressed, it impeded cell growth more seriously than *D484K*—a sub-lethal phenotype was observed (Fig. 5a). The expression levels of both mutants were similar (Supplementary Fig. 6). One explanation of the increased toxicity could be that the D484K variant is not fully activated, and the second substitution F508A further releases the M-domain-driven repression and increases the hyperactivity. However, when we measured the activity of the purified Hsp104 D484K F508A, it had the same ATPase activity (Supplementary Fig. 7a) and it refolded aggregated luciferase and GFP without Hsp70 with the same efficiency as Hsp104 D484K (Fig. 5b). Since disaggregation activity in the absence of Hsp70 was low due to the inhibitory effect of ADP [32], we also measured GFP disaggregation in the presence of the ATP regeneration system (Supplementary Fig. 7b). In this case, the activity of Hsp104 D484K F508A was much higher due to the lack of ADP inhibition but did not exceed than that of Hsp104 D484K. Similarly, in the BLI assay, Hsp104 D484K F508A alone did not show stronger binding to the aggregate than Hsp104 D484K (Fig. 5d). Apparently, the F508A mutation does not make Hsp104 D484K variant more hyperactive. Analogous experiments carried out in the presence of Hsp70 show that any Hsp104 variant with the F508A substitution is no longer stimulated by addition of the Hsp70 system (Fig. 5c). These results indicate that it is lack of collaboration with Hsp70 that makes the hyperactive Hsp104 D484K F508A more toxic. This suggests that Hsp70 is an important factor that controls the disaggregase and prevents its harmful activity.

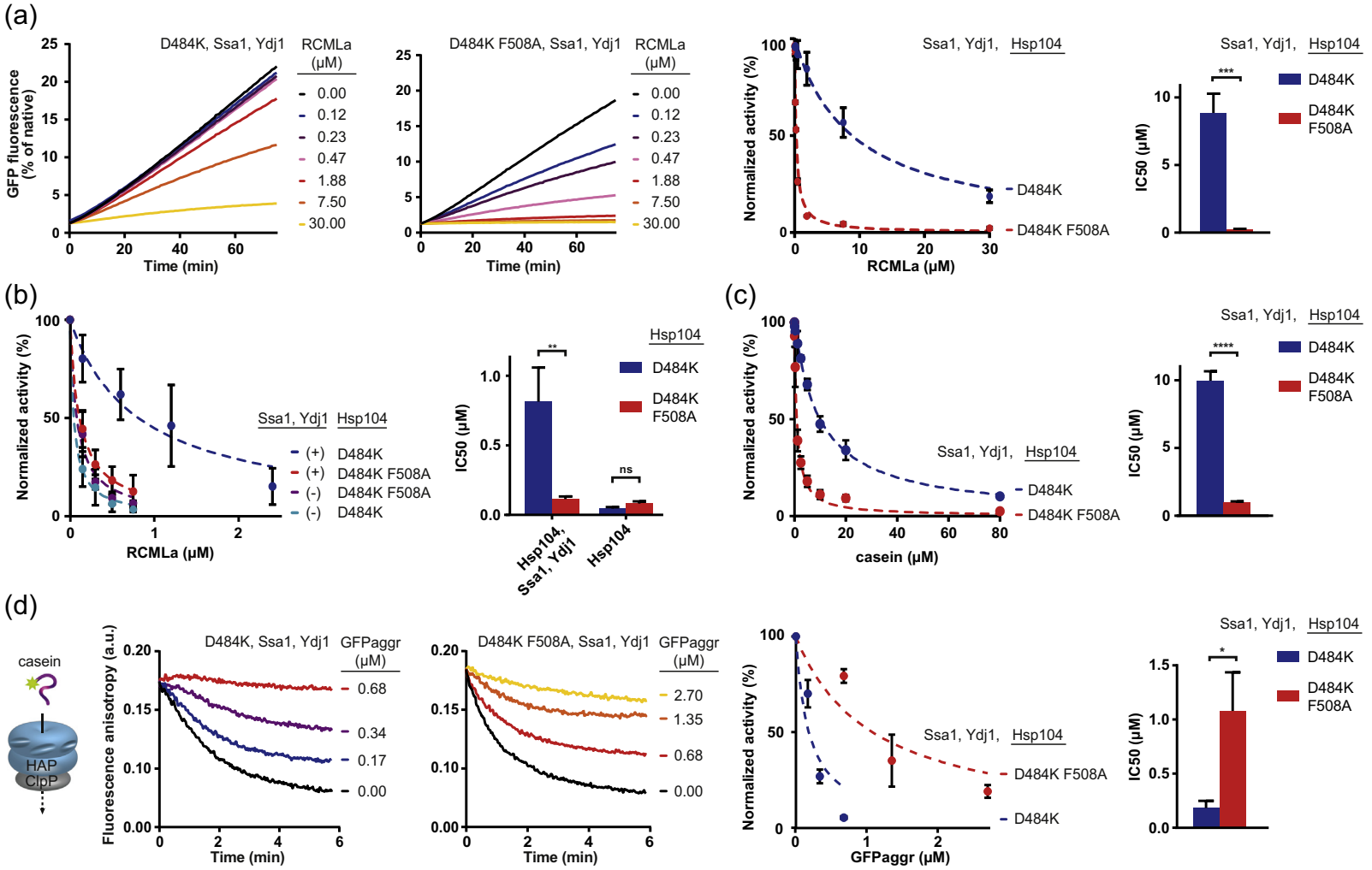
The effect of disrupted interaction with Hsp70 on the substrate selectivity of Hsp104

The toxicity of the hyperactive Hsp104 variants has been associated with unfolding and destabilization of intrinsically disordered proteins that are important for the cell, such as polarisome component, Spa2 [31]. We asked if such side activity of Hsp104 toward non-aggregated proteins can be promoted by a lack of cooperation with Hsp70. Previously, we showed that the support of Hsp70 in protein processing is limited to aggregated substrates [32]. Continuing these studies, we incubated Hsp104 simultaneously with two competing substrates: aggregated GFP and a model disordered protein substrate, reduced carboxymetylated lactalbumine (RCMLa), to assess how cooperation with Hsp70

Fig. 5. Hsp70-dependent recruitment of Hsp104 to aggregates prevents its toxicity. (a) Effect of interaction with Hsp70 on the toxicity of the hyperactive Hsp104 mutant. An equal number of yeast cells W303 *hsp104*Δ with pRS313 plasmid carrying WT and the mutants of the *HSP104* gene were serially diluted, spotted onto SC (-His) plate and grown at 30 °C for 2 days. (b) Refolding of urea denatured luciferase (left panel) by Hsp104 WT, Hsp104 D484K, Hsp104 D484K F508A or Hsp104 F508A (0.5 μM). Refolding of heat-aggregated GFP (right panel) by Hsp104 WT, Hsp104 D484K, Hsp104 D484K F508A or Hsp104 F508A (1 μM). (c) Refolding of urea denatured luciferase (left panel) by Hsp104 WT, Hsp104 D484K, Hsp104 D484K F508A or Hsp104 F508A (0.5 μM) in the presence of Ssa1 (2 μM) and Ydj1 (1 μM). Refolding of heat-aggregated GFP (right panel) by Hsp104 WT, Hsp104 D484K, Hsp104 D484K F508A or Hsp104 F508A (1 μM) in the presence of Ssa1 (1 μM) and Ydj1 (1 μM). Data for luciferase are the mean (±SD) of three independent experiments. (d) Interaction of the hyperactive Hsp104 variants with luciferase aggregates in the absence (left panel) or presence (right panel) of the Hsp70 system. BLI sensor covered with luciferase aggregates was incubated in the presence of 5 mM ATP with or without Ssa1 and Ydj1 (1 and 0.8 μM, respectively) until the binding equilibrium, followed by the addition of Hsp104 WT, Hsp104 D484K, Hsp104 F508A or Hsp104 D484K F508A (to the final concentration of 1 μM).

affects the substrate selectivity of the disaggregase. We used the hyperactive Hsp104 variants, D484K and D484K F508A, to bypass the need for Hsp70 to activate Hsp104, and an ATP regeneration system, to counteract ADP inhibition of disaggregation. With increasing concentration of RCMLa, in the absence of Ssa1 and Ydj1, refolding of aggregated GFP was similarly inhibited for both D484K and D484K F508A (Supplementary Fig. 8a). However, when the Hsp70 system was included, it significantly decreased the




(a)
D484K, Ssa1, Ydj1
RCMLa (μM)
0.00
0.12
0.23
0.47
1.88
7.50
30.00
GFP fluorescence (% of native)
Time (min)
D484K F508A, Ssa1, Ydj1
Ssa1, Ydj1, Hsp104
D484K
D484K F508A
Normalized activity (%)
RCMLa (μM)
IC50 (μM)
(b)
Ssa1, Ydj1 Hsp104
(+) D484K
(+) D484K F508A
(-) D484K F508A
(-) D484K
Hsp104
Hsp104, Ssa1, Ydj1
(c)
casein (μM)
(d)
casein
HAP
ClpP
Fluorescence anisotropy (a.u.)
GFPaggr (μM)
0.68
0.34
0.17
0.00
2.70
1.35
GFPaggr (μM)

degree of inhibition in case of the D484K variant, while the effect of RCMLa on D484K F508A was strong regardless of Hsp70 (Fig. 6a). We observed similar effects when we titrated luciferase aggregates with RCMLa (Fig. 6b) and when we measured GFP disaggregation at the increasing concentrations of another model disordered substrate, α-casein (Fig. 6c; Supplementary Fig. 8b). We also inspected how the presence of GFP aggregates affects Hsp104-dependent processing of α-casein. To monitor translocation of the disordered substrate in real time, we used α-casein labeled with fluorescein (FITC-casein) and Hsp104 variant termed HAP, which is capable of protein degradation in complex with the ClpP protease [45]. When we incubated FITC-casein with either HAP D484K or HAP D484K F508A in the presence of Ssa1, Ydj1 and ClpP, we observed a decrease in fluorescence anisotropy associated with the degradation of FITC-casein and the release of short fluorescein-bound peptides (Fig. 6d). Addition of aggregated GFP to the reaction inhibited the proteolysis of FITC-casein by HAP D484K more efficiently than by HAP D484K F508A (Fig. 6d). From these experiments, we conclude that the effective collaboration with Hsp70 shifts substrate specificity of Hsp104 toward aggregated proteins. This way, the recovery of aggregated proteins is promoted and the unfolding of native disordered proteins is inhibited. The fact that both effects are to the advantage of the cell underlines the critical regulatory role of Hsp70.

Discussion

Hsp104 disaggregase is the central chaperone of the system that counteracts proteotoxicity associated with stress and aging. Despite the critical role of this protein-remodeling machine, there is a trade-off: on one hand, it must be powerful enough to tackle protein aggregates; on the other hand, it needs to be restricted to prevent cell toxicity. Here, we investigated how collaboration with the Hsp70 chaperone at the initial stages of disaggregation allows to maintain this balance by providing effective and precise recognition of an aggregated substrate.

Identification of the Hsp70-interaction site

Hsp70 can be involved in disaggregation either in complex with the disaggregase or by affecting the substrate conformation independently of Hsp104 [6,43]. To reconcile, which function is more important for efficient initiation of disaggregation, we assessed the relevance of direct Hsp70–Hsp104 interaction by disrupting the Hsp70-binding site on the M-domain of Hsp104. We determined the position of the crucial residue basing on sequence alignment of ClpB/Hsp104 proteins from the representatives of all kingdoms of life. This comparison sheds some light on the reported discrepancies between the bacterial and eukaryotic systems, regarding their mechanism of cooperation with Hsp70. In bacteria, but not in yeast, the interaction is mediated by a globally conserved aromatic residue in the position homologous to Y507 in *Sc*Hsp104 (Fig. 2). We established that in yeast the same role is played by the neighboring residue F508, the aromatic character of which is conserved among the Unikonta/Amorphea clade. At the same time, homologous position in Prokaryotes and several remaining groups of Eukaryotes, including Plants and Algae, is occupied by a helix-breaker glycine (Fig. 2). The difference in the orientation of the critical aromatic side chain and the local secondary structure could explain the incompatibility between the systems, likely brought on by mutation in *HSP104* from an ancestor of the Unikonta/Amorphea supergroup. Nevertheless, in the context of the high mobility of the M-domain [10,17,18,25,46] and its variable length among species (Supplementary Fig. 9), the difference in the Hsp70-interaction site in ClpB and Hsp104 is small. Although it cannot be ruled out that other sites could also be important for Hsp70

Fig. 6. Hsp70 shifts substrate preference of Hsp104 toward aggregates. (a) Left panels: Disaggregation and refolding of aggregated GFP (0.35 μM) by Hsp104 D484K or Hsp104 D484K F508A (0.2 μM) variant and Ssa1 (1 μM) and Ydj1 (0.5 μM) in the presence of the indicated concentrations of RCMLa. Right panels: Normalized activity of Hsp104 variants *versus* the concentration of RCMLa. The IC50 values calculated for the reactions are shown on the right. (b) Left panel: Normalized disaggregation and refolding rate of heat-aggregated luciferase (0.22 μM) by Hsp104 D484K or Hsp104 D484K F508A (0.25 μM) variant in the presence of Ssa1 (1 μM) and Ydj1 (1 μM) *versus* the concentration of RCMLa. Right panel: The IC50 values calculated for the reactions. (c) Left panel: Normalized disaggregation rates of aggregated GFP measured as in panel b at the indicated concentrations of α-casein. Right panel: The IC50 values calculated for the reactions. (d) Left panels: Proteolysis of FITC-casein by ClpP (0.4 μM) and HAP variants: D484K or D484K F508A (0.2 μM) in the presence Ssa1 (0.5 μM) and Ydj1 (0.5 μM), and the indicated concentrations of GFP aggregates. Proteolysis was measured by following changes in fluorescence anisotropy. Right panels: Normalized proteolytic activity of HAP D484K and HAP D484K F508A against α-casein *versus* the concentration of GFP aggregates. The IC50 values are shown on the right. (a–c) Dashed lines represent the *[Inhibitor] versus response* model from the Graphprism software fitted to the experimental data from three experiments using least square fitting. The IC50 values calculated for the reactions are shown on the right. Two-tailed *t*-test, $^{****}P \leq 0.0001$, $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, ns $P > 0.05$ ($n = 3$). Data are mean ± s.d.

binding [47], our results support a generally conserved mechanism of cooperation between the chaperone partners [33].

Interaction with Hsp70 makes the disaggregase more efficient

The studies of the hyperactive Hsp104 and ClpB variants showed that Hsp70 binding to the M-domain drives the disaggregase into a highly active mode, characterized by higher affinity for protein substrates [17,18,28,29,34]. However, due to the considerable level of residual ADP in cytoplasm, this allosteric activation, although necessary, is not sufficient to fully engage Hsp104 in disaggregation, as even the hyperactive variants strongly depend on Hsp70 for aggregate binding [28,32]. In this study, we also observed strong dependence on Hsp70 for both WT and D484K variants. In contrast, Hsp104 F508A and its more hyperactive D484K F508A variant do not show stronger aggregate binding in the presence of Hsp70 (Fig. 5d), which indicates that the physical interaction between the chaperone partners through phenylalanine 508 is critical to stabilize the complex between Hsp104 and aggregated substrate.

We were able to monitor the formation of the disaggregation initiation complex by adopting BLI for the real time aggregate-binding analysis. This technique allowed to verify that Hsp104 F508A variant does not bind to the Hsp70-covered aggregate surface. The obtained results are in agreement with the *in vivo* localization of the Hsp104 F508A–mCherry protein under heat stress (Fig. 4c). As a result of disrupted substrate binding, Hsp104 F508A possesses very limited disaggregation activity in the presence of Hsp70, regardless of the degree of hyperactivity. Accordingly, the disaggregase that is not regulated by Hsp70 does not provide thermotolerance in yeast.

The BLI technique also allowed us to examine the effect of the Hsp104-independent activity of the Hsp70 system on disaggregase binding to the aggregate surface. Hsp70 could facilitate Hsp104 binding to aggregates by their initial remodeling in a way that increases aggregate surface or that makes chaperone-binding sites more accessible. Such mechanism can be suggested based on the observation that the Hsp104 F508A reactivates proteins more rapidly when the aggregates have been pre-incubated with the Hsp70 system (Supplementary Fig. 4). Using BLI, we monitored sequential binding of individual chaperones to the aggregate surface, and we did not observe any positive effect of Hsp70 on aggregate binding by Hsp104 F508A (Figs. 4a, b and 5d), as well as no stimulation in case of the heterologous counterparts (Fig. 1b). This, along with the *in vivo* results, indicates that the independent aggregate-remodeling activity of Hsp70 does not play major role in Hsp104 recruitment for disaggregation.

Interaction with Hsp70 makes the disaggregase less toxic

The Hsp70–Hsp104 interaction, involving F508, is responsible for both Hsp104 activation and association to the Hsp70-bound aggregate. While it has been clear that the allosteric regulation via the M-domain serves to restrict the toxic protein-unfolding activity [17,18], we observed that Hsp70-dependent sequestration of Hsp104 on the aggregate surface also plays a protective role in the cell. The hyperactive Hsp104 D484K protein, which retains the ability to bind Hsp70, was significantly less toxic that the D484K F508A mutant—hyperactive to the same degree but completely unleashed from the control of Hsp70. Apparently, the toxicity of Hsp104 variants is determined by two factors: hyperactivity and the ability to form a complex with Hsp70. This is important in the light of the ongoing search for the therapeutic application of hyperactive Hsp104 variants in neurodegenerative diseases [48–50]. Each of these two factors could be selected for individually, to generate less toxic Hsp104 variants with higher protein-remodeling activity, tailored to Hsp70 from a target organism.

Hsp104 toxicity is believed to result from intensified unfolding and degradation of non-aggregated proteins with disordered regions that are recognized by the disaggregase [28,30,31,51,52]. Higher toxicity of the unleashed Hsp104 D484K F508A mutant relatively to Hsp104 D484K suggests that Hsp70 binding prevents from processing of undesired protein substrates. With the *in vitro* competition assay, we show that in the presence of both aggregated and non-aggregated misfolded proteins, the ability to collaborate with Hsp70 shifts substrate specificity toward aggregates (Figs. 6,7).

How does Hsp70 provide such specificity, although it interacts with both kinds of substrates? Non-aggregated disordered proteins might be sterically unfit to provide binding sites suitable for the Hsp70–Hsp104 complex in a way that binding of Hsp70 alone shields them from processing by Hsp104. Accordingly, the disaggregase might require at least two properly arranged binding sites to form a stable complex with an aggregate, for example, one direct and one through Hsp70 (Fig. 7). Furthermore, due to very low affinity for the chaperone partner [26,53,54], ClpB/Hsp104 could require multiple aggregate-bound Hsp70 molecules to productively associate with the substrate [8,47]. In agreement, our results show that Hsp104 activity is highly sensitive to incorporation of subunits with disrupted Hsp70-binding site (Fig. 3c) and similar behavior has been reported for ClpB [18,34]. The distribution of the chaperone binding sites at the surface of the protein substrate is a plausible criterion

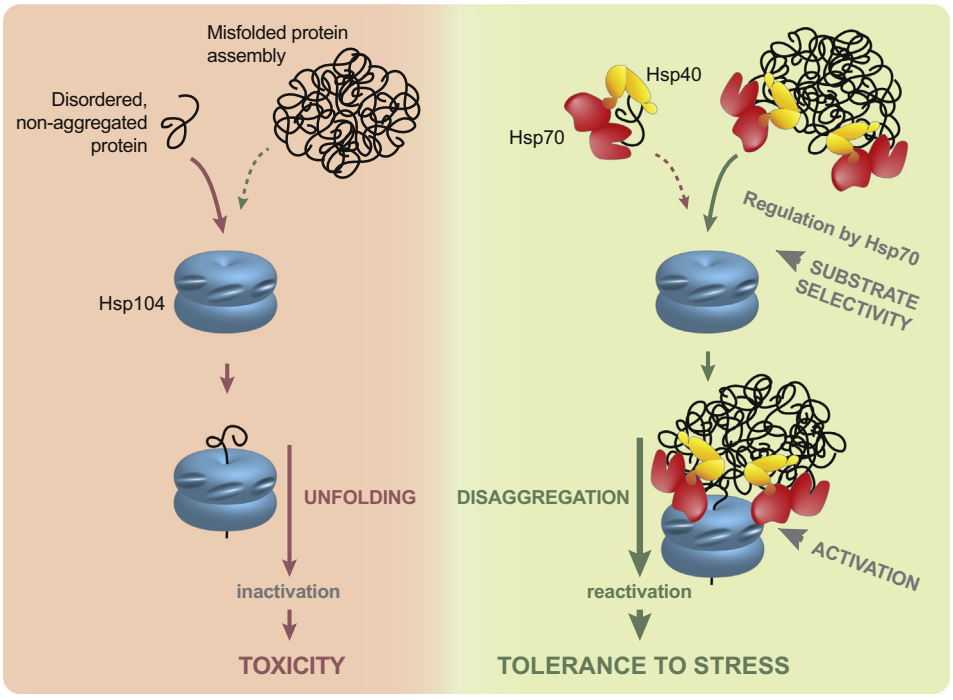


Fig. 7. Interaction with Hsp70 plays dual role in Hsp104 regulation. Hsp104 is capable of remodeling of disordered/misfolded proteins that are either in a non-aggregated form or assembled into insoluble aggregates. Processing of the latter substrates allows their reactivation and removal of toxic aggregates (e.g., amyloid), which is beneficial to the cell. In contrast, unfolding of proteins with disordered regions within their native structure temporarily inactivates them and increases the chance of their misfolding and degradation. In case of proteins with important cellular functions, such promiscuous Hsp104 activity might be toxic. Hsp70 via the direct interaction with Hsp104 favors recognition of aggregates and inhibits Hsp104 binding to non-aggregated disordered proteins. When the disaggregation initiation complex is being formed, the same interaction results in allosteric activation of Hsp104. These two mechanisms make the Hsp104-dependent quality control more selective, safe and efficient.

for selectivity of the disaggregation initiation complex, yet it requires further research to understand this aspect of selective substrate recognition by the Hsp104–Hsp70 bi-chaperone system.

Materials and Methods

Strains and plasmids

All plasmids as well as *Escherichia coli* and yeast strains used in this study are listed in Supplementary Tables 1–4. Hsp104 GFP and mCherry C-terminal tagging was performed in *W303-1B Saccharomyces cerevisiae* strain by chromosomal integration of PCR-amplified cassette replacing the chromosomal copy of HSP104 according to Winkler *et al.* [36]. The construction of cassettes for chromosomal integration was performed by FastCloning according to Li *et al.* [55]. Briefly, PCR-amplified fragments of plasmids *pET5a–HSP104* or *pET5a–hsp104(F508A)* and *pFA6a-link–mCherry–SpHis5* or *pFA6a–GFP(S65T)–His3MY6* were digested with DpnI and mixed at 1:1 ratio and transformed into *E. coli* DH5α super-competent cells, which were subsequently spread on an appropriate selection medium and incubated at 37 °C. Plasmid DNA was isolated and sequenced to verify the correctness of the *HSP104GFP(S65T)-His3MX6* and *HSP104-link–mCherry–SpHis5m* constructs.

For chromosomal modification, we amplified *HSP104–mCherry–HIS3* and *HSP104–GFP–HIS3* sequences with PCR using primers with additional genome-targeting sequences for homologous recombination (primer fwd: GCTGATTTAAGGTACTTCG CCATCCCAGATATCAAAAAGCAAATCG, primer revGFP: CTGATTCTTGTTCGAAAGTTTTTAAAAA TCACACTATATTAAATCACATCAAAACAC, primer_-revmCherry:_CTGATTCTTGTTCGAAAGTTTT-TAAAAATCACACTATATTAAACAGTATAGCGAC-CAGCATTCACATACG). W303-1B cells were transformed with the amplified fragments and spread on SC-His selective medium. Chromosomal modifications were confirmed by sequencing (*HSP104–GFP*, *HSP104–mCherry*, *hsp104(F508A)–GFP* and *hsp104 (F508A)–mCherry*). Synthesis of fusion proteins in yeast cells was additionally confirmed by Western blotting using anti-Hsp104, anti-GFP or anti-mCherry antibodies (Supplementary Figs. 5c and 10). Point mutations were introduced with PCR site-specific

mutagenesis (Qiagen) and confirmed by sequencing of the *HSP104* gene. Plasmids were introduced into *S. cerevisiae* strain W303*hsp104*Δ by lithium acetate transformation.

Proteins

Published protocols were used for the purification of Hsp104 and its variants, and Ydj1 [28], GFP [56], Ssa1 [57], and ClpP [58]. Luciferase with C-terminal HisTag was expressed from the *pOPINA-Luciferase* plasmid using IPTG induction following Ni-NTA purification. HAP variant of Hsp104 (G739I S740G K741F T746A) was expressed from *pET24a–HAP* plasmid, a gift from B. Bukau (University of Heidelberg). RCMLa was prepared as described [59]. α-casein, FITC-α-casein, α-lactalbumin, lactic dehydrogenase, pyruvate kinase and creatine kinase were purchased from Sigma. Untagged luciferase was purchased from Promega. All given protein concentrations refer to monomer.

Sequence analysis

Amino acid sequence of Hsp104 from *S. cerevisiae* was used as a query to identify putative homologs in the selected genomes (Supplementary Fig. 9) using TBLASTN (NCBI). Sequences were aligned using MAFFT version 7 online server [60], using BLOSUM62 substitution matrix with L-INS-I method [61].

Refolding of heat-aggregated GFP

GFP renaturation assay was performed as described previously [56]. Briefly, GFP (2 mg/ml) was thermally inactivated at 85 °C for 15 min. The reactivation reaction was carried out at 25 °C in the renaturation buffer A [28 mM Tris–HCl (pH 7.8), 7% (v/v) glycerol, 60 mM potassium glutamate, 7 mM DTT, 15 mM MgOAc, 10 mM ATP]. Renaturation was initiated by adding the chaperone proteins: Hsp104 WT and its variants (1 μM), Ssa1 (1 μM) and Ydj1 (1 μM) when indicated. ATP regeneration system comprising of 0.1 mg/ml creatine kinase and 20 mM creatine phosphate was added in experiments presented in Fig. 6 and Supplementary Figs. 2b, 4, 7b and 8. GFP fluorescence was detected in a JASCO FP-8000 Fluorescence Spectrometer or in a Beckman Coulter DTX880 microplate reader. The curves presented in the figures are representative for at least three experiments.

Refolding of urea denatured luciferase

Luciferase (1.7 mg/ml) was incubated for 15 min at 48 °C in buffer C [40 mM Tris–HCl (pH 7.5), 150 mM KCl, 1 mM DTT, 15 mM MgOAc] supplemented with 6 M urea. Denatured luciferase was subsequently 40-fold diluted into the buffer C without denaturant. The refolding reaction was performed at 25 °C in the buffer C supplemented with 10 mM ATP and chaperones (0.5 μM Hsp104, 2 μM Ssa1, 1 μM Ydj1). ATP regeneration system comprising of 0.1 mg/ml creatine kinase and 20 mM creatine phosphate was added in experiments presented in Fig. 6b. In the mixing experiment presented in Fig. 3c, the total concentration of Hsp104 and Hsp104 F508A was 0.5 μM. Mixing ratios are indicated in figure legend. As a control, Hsp104 was mixed with buffer instead of Hsp104 F508A. Luciferase during refolding was present at 65 nM concentration. The luciferase activity was measured in a Sirius Luminometer using Luciferase Assay system (Promega). The activity of renatured luciferase was compared with the native protein (activity of native enzyme set as 100%). Each experiment was repeated at least three times. Data are the mean (±SD).

Thermotolerance assay

Yeast cells *W303 hsp104*Δ transformed with pRS313, or pRS313 *HSP104*, or pRS313 *hsp104 F508A* plasmid were grown at 30 °C in SC(-His) medium. Heat shock was performed on exponentially growing cells adjusted to an OD_{600} of 0.4. Cells were pre-incubated at 37 °C for 30 min, and then incubated with agitation at 50 °C. Aliquots were withdrawn at the indicated time points, and cells were mixed, serially diluted (5-fold each dilution) and spotted on SC(-His) solid media and grown for 2 days at 30 °C.

Toxicity assay

An equal number of yeast cells *W303 hsp104*Δ transformed with the *pRS313* plasmid carrying variants of the *HSP104* gene were grown at 30 °C and serially diluted and spotted onto the SC (-His) plate.

BLI experiments

BLI experiments were performed on BLItz instrument using Dip and Read™ Ni-NTA (NTA) Biosensors (ForteBio) at room temperature with 2000 rpm mixing. Basal anchoring luciferase layer (0.6 mg/ml His-tagged luciferase) was immobilized on the sensor at denaturing conditions in buffer D [50 mM Tris (pH 7.5), 150 mM KCl, 20 mM $MgCl_2$, 2 mM DTT] with 6 M urea for 15 min, following washing with buffer D. Top luciferase aggregate layer was formed by transferring the sensor to a test tube with 0.1 mg/ml luciferase in buffer D and subsequent incubation at 44 °C for 10 min. Next, sensor was transferred back to the BLItz instrument and washed in buffer D for 10 min. Then, binding of chaperone proteins in the concentrations stated in figure legends to such prepared BLI sensor was analyzed according to the scheme in Fig. 1a. The curves presented in the figures are representative for at least three experiments.

Competition experiments

Heat-aggragated GFP (0.35 μM) was refolded in the presence of RCMLa (0–30 μM) or α-casein (0–80 μM) and the following chaperones: Hsp104 variants (0.2 μM), Ssa1 (1 μM) and Ydj1 (0.5 μM). The reaction was performed in buffer B with ATP regeneration system comprising 20 mM creatine phosphate and 0.1 mg/ml creatine kinase at 25 °C. GFP fluorescence was detected in a Beckman Coulter DTX880 microplate reader.

Firefly luciferase (2.22 μM) was denatured for 10 min at 44 °C in buffer E [25 mM Hepes (pH 8.0), 75 mM KCl, 15 mM $MgCl_2$]. It was refolded at a concentration of 0.22 μM in the presence of Hsp104 variants (0.25 μM), Ssa1 (1 μM), Ydj1 (1 μM) and increasing concentrations of RCMLa (0–2.4 μM) in buffer E containing 5 mM ATP and the ATP regeneration system (0.1 mg/ml creatine kinase and 20 mM creatine phosphate) at 25 °C. The measurement was made after 40 min of incubation. This time point was chosen, as we repeatedly observed linear growth of the reactivated luciferase signal to up to 60 min duration of reactivation reaction. Measurements were made using Sirius Luminometer (Berthold).

Proteolysis of FITC-casein (1.8 nM) (Sigma C0528) in the presence of GFP aggregates (0–2.7 μM) was analyzed by fluorescence anisotropy at 30 °C in buffer B supplemented with the ATP regeneration system (0.1 mg/ml creatine kinase and 20 mM creatine phosphate) and 0.5 μM Ssa1, 0.5 μM Ydj1 and 0.4 μM ClpP. The reaction was initiated by the addition of HAP versions of Hsp104 (0.2 μM). Measurements were performed in JASCO FP-8000 Fluorescence Spectrometer.

The disaggregation rates were measured based on initial linear portion of the enzyme reaction. IC50 was calculated by fitting the [Inhibitor] *versus* normalized response model to the data from three experiments using the GraphPrism Software.

Fluorescence microscopy

The yeast cells *W303–hsp104WT–mCherry* and *W303–hsp104F508A–mCherry* were transformed using *pRS306 citrine–luciferase* plasmid [36] (a gift from B. Bukau, University of Heidelberg) and were subsequently spread on an appropriate selection medium (SC-Ura) and incubated at 30 °C for 3–4 days. Next, transformants were transferred to liquid medium and grown overnight at 30 °C. Cell cultures were then diluted into fresh medium (OD = 0.3) and grown for 3 h at 30 °C. Following this, the cultures were transferred to 42 °C or kept at 30 °C for 10 min. Yeast cells were imaged using a confocal laser scanning microscope (Leica SP8X equipped with an incubation chamber for the live analysis) with a 100× oil immersion lens (Leica, Germany). To minimize fluorescent crosstalk during imaging, sequential scanning between lines was used: excitation 587 nm and detection 610–779 nm for mCherry, and excitation 515 nm and detection 529–587 nm for citrine. ImageJ software was used for photograph preparation. No manipulations other than contrast and brightness adjustments for images were applied. Colocalization studies were performed as described in Ref. [36] using ImageJ software for image analysis. mChery fluorescence was observed in 99% of cells expressing *HSP104–mCherry* and 98% of cells expressing *hsp104 F508A–mCherry.* Citrine fluorescence was observed in 97% of cells expressing *HSP104–mCherry* and 99% of cells expressing *hsp104 F508A–mCherry*.

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Appendix A. Supplementary data

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Abbreviations used:
BLI, Bio-Layer Interferometry; RCMLa, reduced carboxymetylated lactalbumine; GFP, green fluorescent protein.

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