

# Role of sHsps in organizing cytosolic protein aggregation and disaggregation

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**Abstract** Small heat shock proteins (sHsps) exhibit an ATP-independent chaperone activity to prevent the aggregation of misfolded proteins *in vitro*. The seemingly conflicting presence of sHsps in insoluble protein aggregates in cells obstructs a precise definition of sHsp function in proteostasis networks. Recent findings specify sHsp activities in protein quality control systems. The sHsps of yeast, Hsp42 and Hsp26, interact with early unfolding intermediates of substrates, keeping them in a ready-to-refold conformation close to the native state. This activity facilitates substrate refolding by ATP-dependent Hsp70-Hsp100 disaggregating chaperones. Hsp42 can actively sequester misfolded proteins and promote their deposition at specific cellular sites. This aggregase activity represents a cytoprotective protein quality control strategy. The aggregase function of Hsp42 controls the formation of cytosolic aggregates (CytoQs) under diverse stress regimes and can be reconstituted *in vitro*, demonstrating that Hsp42 is necessary and sufficient to promote protein aggregation. Substrates sequestered at CytoQs can be dissociated by Hsp70-Hsp100 disaggregases for subsequent triage between refolding and degradation pathways or are targeted for destruction by selective autophagy termed proteophagy.

**Keywords** Chaperone · Small heat shock protein · Holdase, aggregase · Protein aggregation · Protein disaggregation

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## Introduction

A broad spectrum of stress conditions and physiological imbalances promotes protein misfolding, disrupting cellular proteostasis. Misfolded proteins typically expose hydrophobic patches, which target them to cellular refolding and degradation machineries. Refolding activities are executed by ATP-dependent chaperones mainly of the Hsp60, Hsp70, Hsp90, and AAA+ families, while proteolysis involves ATP-dependent proteases including the ubiquitin-proteasome system (UPS) and autophagic pathways. The importance of these activities in protein quality control networks is well defined. Much less is known about the role of small heat shock proteins (sHsps) in proteostasis networks and the connection of this chaperone family to refolding and degrading activities. sHsps were originally shown to exhibit an ATP-independent “holdase” activity *in vitro*, preventing the formation of large protein aggregates by binding to misfolded protein species (Haslbeck et al. 2005a; Jakob et al. 1993). This activity seemingly conflicts with the observation that *in vivo* sHsps are mostly found as integral parts of protein assemblies and aggregates. Recent findings reveal novel activities and characteristics of sHsps, showing that they can actively sequester proteins upon initial unfolding for deposition at specific cellular sites in near-native states. This review discusses the novel function of sHsps as cellular “aggregases” and links this activity to downstream-acting protein quality control pathways.

## sHsps bind misfolded proteins to form sHsp/substrate complexes

sHsps represent the most diverse class among the conserved families of molecular chaperones. They share a  $\alpha$ -crystallin domain (ACD) of 90 amino acids’ length as unifying element (Basha et al. 2012; Haslbeck et al. 2005a). The ACD is flanked by N- and C-terminal extensions (NTEs, CTEs),

which are variable in length and sequence and provide the basis for sHsp diversity. Most sHsps form dimers, which are in dynamic equilibrium with higher oligomers forming structures of globular shape. Only recently, sHsps were characterized which constitutively form dimers (Basha et al. 2013; Bepperling et al. 2012). As for their primary sequence, sHsps exhibit a great variability in oligomerization states as oligomers differ in subunit numbers (12 to >48) and are frequently polydisperse.

In vitro sHsps were shown to prevent the formation of turbid, insoluble protein aggregates (Jakob et al. 1993), which originally led to their definition as “holdases”. This term was used in distinction to “foldases” which have the capability of (re) folding unfolded or aggregated substrates, such as the Hsp60, Hsp70, and Hsp90 chaperones (Hartl et al. 2011). In vitro, sHsps bind to aggregation-prone misfolded proteins in an ATP-independent manner, to form sHsp/substrate complexes, which are smaller than protein aggregates formed in the absence of sHsps. The final size of sHsp/substrate complexes largely depends on the sHsp-substrate ratio; increasing sHsp amounts lead to reduced complex sizes. sHsp/substrate complexes form a continuum of diverse complex sizes with remarkably high heterogeneity. A 1:1 ratio of sHsp/substrate can result in the formation of more than 300 distinct assemblies (Stengel et al. 2010).

The capacity of sHsps for substrate interactions is very high as they can bind to an equal weight of misfolded proteins. This is in line with the fact that sHsps do not harbor a single defined substrate-binding site, but all domains contribute to substrate interactions, suggesting multivalent interactions. The intrinsically disordered NTEs provide the majority of interaction sites (Fu et al. 2013; Haslbeck et al. 2004; Jaya et al. 2009), but exposed hydrophobic sites in ACD and also the flexible CTE are able to contact substrates as well (Fu et al. 2013; Jaya et al. 2009; Ungelenk et al. 2016).

### **sHsps bind early-unfolding intermediates and keep substrates in near-native states**

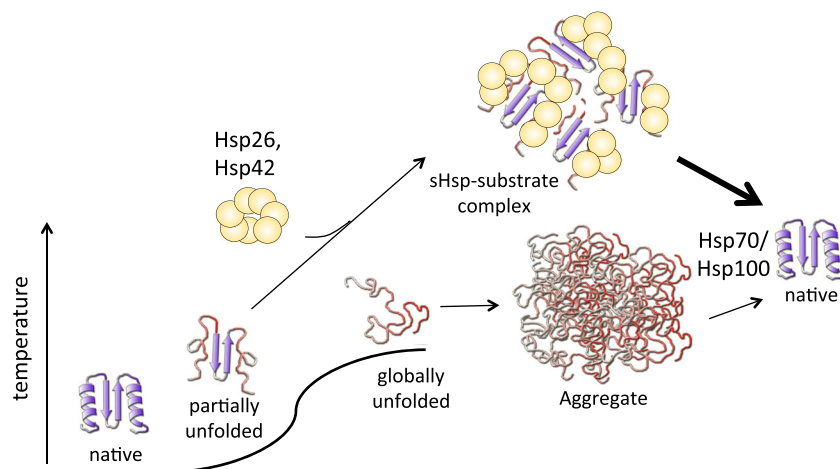
How sHsps interact with substrates is the key to understanding their mode of action in proteostasis. To answer this question, it is necessary to determine the consequences of sHsp binding on substrate conformation by comparing the conformations of a substrate when bound to sHsps and when aggregating upon unfolding in the absence of sHsps. A recent study determined the structural flexibility of both substrate states (aggregated vs. bound to sHsps) by amide hydrogen exchange (HX) using thermolabile, dimeric malate dehydrogenase (MDH) as substrate (Ungelenk et al. 2016). HX determines the accessibility of amide protons of the peptide backbone and thereby reports on the structural flexibility of proteins at the peptide level. Aggregated MDH showed strongly increased HX throughout its primary sequence, indicating that it is globally unfolded and

does not retain substantial secondary structure. Binding of MDH to the yeast sHsps, Hsp26 and Hsp42, resulted in increased protection of MDH that was particularly pronounced at the MDH core including the dimer interface (Ungelenk et al. 2016). The presence of a native-like core structure of MDH was also found when the substrate was bound to plant sHsps, suggesting a conserved principle of sHsp-MDH interaction (Cheng et al. 2008). Notably, the conformations of MDH peptides are not uniform in sHsps/MDH complexes but exist as mixtures of native-like and aggregate-like states (Ungelenk et al. 2016). The conformational heterogeneity is largest at substoichiometric sHsp-MDH ratios, conditions that do not allow binding of all MDH molecules to sHsps. Increasing sHsp levels shift MDH peptide conformations towards native-like states, suggesting that sHsps bind to early-unfolding intermediates and protect bound MDH from further unfolding. This model demands for initial unfolding of peripheral structural elements in the MDH dimer. Consistent with this assumption, exposed C-terminal  $\alpha$ -helices of the MDH dimer still showed increased structural flexibility when bound to sHsps and were identified as dominant interaction site of yeast Hsp26 (Ungelenk et al. 2016). This binding mode also impacts on the overall architecture of the sHsp-substrate assemblies, as the trapped MDH substrates are kept well separated from each other, whereas MDH interactions are tighter in the aggregated state (Fig. 1).

Can the principles of sHsp-substrate interaction derived from analysis of the MDH model substrate be generalized? Findings based on diverse techniques and use of alternative model substrates are in support. Citrate synthase retains its dimeric state when bound to pea Hsp18.1 as shown by mass spectrometry analysis (Stengel et al. 2012). Yeast Hsp42 interacts with native-like states of maltose-binding protein as shown by optical tweezer experiments at the single molecule level (Ungelenk et al. 2016). The ability of sHsps to associate to early unfolding intermediates of substrates and to preserve their structure thus seems to represent a conserved feature (Fig. 1). Notably, sHsp binding to substrates during stress conditions has been initially suggested to create a reservoir of proteins that are competent for refolding upon return to physiological growth conditions (Ehmsperger et al. 1997; Haslbeck et al. 2005a; Kampinga et al. 1994; Lee et al. 1997). The recent findings that sHsps trap substrates in minimally misfolded, near-native conformations are in accordance with and further specify the original model. It should be noted that these findings do not exclude that sHsps may in addition associate with other, more unfolded conformers of protein substrates.

### **sHsps facilitate substrate refolding by disaggregating chaperones**

While sHsps are described to efficiently prevent the formation of insoluble, turbid protein aggregates in vitro, they associate



**Fig. 1** The yeast sHsps, Hsp26 and Hsp42, sequester substrates in near-native states. Without sHsps, substrates globally unfold and form tight protein aggregates. In presence of sHsps (Hsp26, Hsp42), early unfolding intermediates of substrates are sequestered within sHsp/substrate complexes. The sHsps preserve a native-like core structure of bound

substrates and increase the distance between non-native protein molecules keeping substrates separated. These features contribute to facilitated substrate refolding by disaggregating Hsp70-Hsp100 chaperones

with misfolded proteins to become an integral part of large aggregates in vivo (Arrigo et al. 1988). In *Escherichia coli*, sHsps co-sequester with recombinant proteins upon overproduction in inclusion bodies and were therefore termed *i*-nclusion *b*ody-associated *p*roteins IbpA and IbpB (Allen et al. 1992). *E. coli* IbpA/IbpB also represents the most abundant single species of protein aggregates formed during heat stress (Laskowska et al. 1996; Mogk et al. 1999). Quantitative coaggregation of sHsps with misfolded proteins has been also described in heat-stressed *Arabidopsis thaliana* (Lee et al. 2005) and in *Caenorhabditis elegans* during aging (Walther et al. 2015). The evolutionary conserved presence of sHsps in large protein aggregates in vivo indicates that sHsp function in proteostasis networks goes beyond the aggregation preventing holdase activity proposed earlier. As aggregated proteins are solubilized during stress recovery periods by cellular disaggregases, the activity of sHsps is functionally connected to protein disaggregation.

A link of sHsps to protein disaggregation processes became evident by genetic analysis, showing that Hsp16.6, the sHsp of *Synechocystis* sp., is crucial for thermotolerance development (Giese and Vierling 2002). Acquired thermotolerance describes the ability of cells to survive the transient exposure to lethal temperatures upon pre-adaptation by a mild heat shock. This process is ultimately linked to the heat inducible capability to solubilize aggregated proteins, executed by the Hsp70-Hsp100 bichaperone system in bacteria, unicellular eukaryotes, and plant cells (Queitsch et al. 2000; Sanchez and Lindquist 1990; Squires et al. 1991). A role of *Synechocystis* Hsp16.6 in protein disaggregation was further supported by noticing synthetic sickness upon additionally deleting the *clpB* gene, encoding for the AAA+ disaggregase Hsp100 (Giese and Vierling 2002). Stress-sensitive

phenotypes have been meanwhile described for some sHsp knockouts in diverse species (Krajewski et al. 2014; McLoughlin et al. 2016) but they are often not apparent for other sHsps mutants, an observation that can be explained by compensatory proteostasis activities. Indeed, *E. coli* IbpA/IbpB function becomes important at increased temperatures only upon simultaneous Hsp70 (DnaK) depletion, and this effect was aggravated upon additional deletion of *clpB* (Mogk et al. 2003a). These genetic findings suggest that sHsps form a functional triade with the disaggregating Hsp70 and Hsp100 chaperones, and that sHsp function becomes particularly important during severe stress conditions or upon limiting the cellular disaggregation potential.

In vitro analyses of substrate refolding from a sHsp-bound state confirmed the critical role of sHsps in protein disaggregation. Upon association with sHsps, misfolding proteins become trapped and do not efficiently dissociate spontaneously, and hence do not refold to the native state (Friedrich et al. 2004; Haslbeck et al. 2005b; Mogk et al. 2003b). In this sense, sHsp/substrate complexes can be described as protein aggregates, yet they differ strongly in size, composition, and conformational state of the substrate. The dissociation of substrates from sHsp/substrate complexes requires force generation, which is executed by the ATP-empowered disaggregation activities of Hsp70 and Hsp100 chaperone machines (Lee and Vierling 2000; Mogk et al. 2003b; Nillegoda et al. 2015). Hsp70 has a key function in initiating substrate dissociation and refolding from a sHsp-bound state, and this role cannot be taken over by other chaperone systems including Hsp60 (GroEL) (Veinger et al. 1998). An activity of proteolytic systems employing ATP-dependent AAA+ “unfoldases”, including the 26S proteasome, has not been implicated either, underlining the tight connection between sHsps and Hsp70

chaperones. The stability of sHsp/substrate complexes and the dependence on Hsp70 for substrate dissociation conceivably have an additional physiological role as they impose a tight control of substrate release restricted to favorable folding conditions at which Hsp70 function is available. The dominant role of Hsp70 might also go beyond the dissociation of sHsp/substrate complexes. This chaperone might also direct dissociated substrates towards refolding rather than degradation pathways.

How sHsps ensure specificity towards Hsp70 chaperones is an important, yet still unresolved question. sHsps might attract Hsp70 through direct interactions, but evidence for physical contacts are missing. Furthermore, sHsps and Hsp70s, originating from different species efficiently cooperate in vitro (Bepperling et al. 2012; Friedrich et al. 2004; Giese and Vierling 2002; Mogk et al. 2003b). The absence of any species-specific cooperation argues against a key role of sHsp-Hsp70 interactions in conferring Hsp70 preference. Notably, BAG3, a nucleotide exchange factor of Hsp70, functions as a scaffold protein for formation of Hsp70-sHsp complexes in mammalian cells (Carra et al. 2008). BAG3 binds to the ATPase domain of Hsp70 via its BAG domain and to a hydrophobic groove in the  $\alpha$ -crystallin of sHsps via two IPV motifs (Fuchs et al. 2009; Rauch et al. 2016). These modular features enable BAG3 to link Hsp70 and sHsp chaperones, leading to increased chaperone activity (Rauch et al. 2016). BAG3 homologs do not exist in bacteria and yeast. Whether these organisms encode for adaptor proteins with similar activities is currently unknown.

sHsp incorporation into protein aggregates affects protein disaggregation in two ways. First, it increases the kinetics and yields of substrate refolding by the Hsp70-Hsp100 bichaperone system (Cashikar et al. 2005; Haslbeck et al. 2005b; Mogk et al. 2003b; Ratajczak et al. 2009). Second, it enables the Hsp70 system on its own, in the absence of the Hsp100 disaggregase, to partially (bacterial DnaK) or more efficiently (human Hsp70) refold bound substrates, thereby expanding the Hsp70 substrate spectrum (Mogk et al. 2003b; Nillegoda et al. 2015; Rampelt et al. 2012). In agreement with these in vitro findings, protein disaggregation is delayed in sHsp mutant cells in yeast and *E. coli* cells (Cashikar et al. 2005; Mogk et al. 2003a; Ungelenk et al. 2016).

How do sHsps facilitate disaggregation by Hsp70-Hsp100 chaperones? The coaggregation of sHsps with misfolded proteins might decrease aggregate size, thereby enlarging the aggregate surface area accessible for binding of Hsp70 chaperones. The recent findings that sHsps keep sequestered substrates apart and stabilize them in near-native states (Ungelenk et al. 2016) offer alternative explanations. The disentanglement of substrates is easier from sHsp assemblies as compared to that of protein aggregates formed without sHsps, presumably because the number of intermolecular

interactions between substrate molecules is reduced. Additionally, refolding processes might be facilitated as sequestered substrates are kept in a ready-to-refold state close to their native conformation (Fig. 1).

### Sequestration of misfolded proteins as protective strategy

The coaggregation of sHsps with misfolded proteins is employed to actively sequester substrates and promotes their deposition in large cellular assemblies (Escusa-Toret et al. 2013; Miller et al. 2015b; Saarikangas and Barral 2015; Shiber et al. 2013; Song et al. 2014; Specht et al. 2011). This concept is changing our view on protein aggregation, which so far was described as an uncontrolled disastrous process resulting from proteostasis collapse only. The traditional view was initially challenged by the identification of specific deposition sites for misfolded proteins in a variety of cells, implying that aggregation is an organized process (Johnston et al. 1998; Kaganovich et al. 2008; Tyedmers et al. 2010). The identification of cellular factors which act as aggregases (or sequestrases) to promote and control the aggregation process argues further against protein aggregation being simply a consequence of misfolded protein overload (Johnston et al. 1998; Kawaguchi et al. 2003; Malinowska et al. 2012; Miller et al. 2015a; Specht et al. 2011). The organized and active sequestration of misfolded proteins was therefore suggested to represent a novel, third strategy of proteostasis networks (Chen et al. 2011). This strategy provides cytoprotective functions upon protein-folding stress in yeast cells (Escusa-Toret et al. 2013; Ungelenk et al. 2016). Cytoprotection may involve several mutually non-exclusive effects. First, controlled deposition of soluble misfolded proteins can reduce their cytotoxicity (Arrasate et al. 2004; Cheng et al. 2007; Cohen et al. 2006; Cohen et al. 2009; Walther et al. 2015; Wolfe et al. 2013). Second, it might prevent exhaustion of the protein quality control machinery during protein folding stress, by confining the sticky surfaces of misfolded proteins. Third, concentrating misfolded proteins at specific deposition sites could facilitate subsequent protein refolding by chaperones or clearance by the UPS or autophagy. Fourth, targeting of misfolded proteins to deposition sites could allow for asymmetric inheritance of damaged proteins during cell division, leading to the formation of aggregate-free daughter cells (Aguilaniu et al. 2003; Liu et al. 2010; Rujano et al. 2006; Spokoini et al. 2012; Zhou et al. 2014).

### Role of Hsp42 in protein aggregation in the yeast cytosol

Main progress in understanding mechanism and physiological impact of organized protein aggregation was achieved in the model organism *Saccharomyces cerevisiae*. Here, protein sequestration and formation of protein aggregates at specific cellular sites are typically monitored by the use of fluorescent



misfolded reporter proteins (e.g., mcherry-VHL, GFP-Ubc9<sup>ts</sup>), which form fluorescent foci during physiological stress. These foci differ in cellular localizations and characteristics (Kaganovich et al. 2008). The original nomenclature for deposits JUNQ (juxtannuclear quality control compartment) and IPOD (insoluble protein deposit) (Kaganovich et al. 2008) was modified and expanded by further work. JUNQ was later demonstrated to reside inside the nucleus and was therefore termed INQ (intra-nuclear quality control compartment) (Gallina et al. 2015; Miller et al. 2015a). Moreover, apart from IPOD and INQ (JUNQ), additional stress-induced aggregates were described to exist in the yeast cytosol. These deposits were referred to as peripheral aggregates (Specht et al. 2011), stress foci (Song et al. 2014; Spokoini et al. 2012), Q-bodies (Escusa-Toret et al. 2013), or APOD (Saarikangas and Barral 2015). As all of these aggregates reside in the cytosol and depend on Hsp42 for formation, we simplify the currently non-uniform nomenclature in this review and refer to them as CytoQ (cytosolic quality control compartment) (Miller et al. 2015a). We define IPOD as an Hsp42-independent aggregate form mainly of amyloidogenic proteins including the yeast prions Rnq1 and Sup35 (Kaganovich et al. 2008; Kumar et al. 2016).

CytoQ and INQ formation in yeast is controlled by the compartment-specific cellular aggregases, Hsp42 and Btn2. Btn2 is strongly induced by heat and is essential for INQ formation under physiological stress conditions (Gallina et al. 2015; Malinovska et al. 2012; Miller et al. 2015a). It harbors a nuclear localization sequence and upon stress treatment, a major fraction of cellular Btn2 colocalizes with INQ (Malinovska et al. 2012; Miller et al. 2015a). The constitutively expressed sHsp Hsp42 is required for CytoQ formation at physiological heat-stressed conditions (e.g., 38 °C) (Escusa-Toret et al. 2013; Malinovska et al. 2012; Miller et al. 2015a; Saarikangas and Barral 2015; Shiber et al. 2013; Song et al. 2014; Specht et al. 2011). Hsp42 is excluded from the nucleus, explaining its compartment-specific function in misfolded protein sequestration in the cytosol and its exclusive colocalization with CytoQ (Malinovska et al. 2012; Specht et al. 2011). Artificial targeting of Hsp42 to the nucleus restores INQ formation in *btn2*Δ cells, demonstrating that its aggregase activity as such is compartment-independent (Miller et al. 2015a).

Hsp42 has been shown to organize the sequestration of diverse substrates under different stress regimes, including heat stress, proteasome inhibition, cellular quiescence, and cellular aging (Table 1). The role of Hsp42 in these processes is specific and cannot be taken over by Hsp26, the second yeast sHsp. Hsp42 gains its functional specificity as cellular aggregase via its unusual long N-terminal extension (NTE). NTE deletion abrogates CytoQ formation (Marshall et al. 2016; Specht et al. 2011), whereas a hybrid Hsp26 protein harboring the NTE of Hsp42 is largely proficient in CytoQ

formation (Specht et al. 2011). Notably, the long Hsp42 NTE includes a prion-like domain (Alberti et al. 2009). Prion-like domains have been implicated in the formation of large RNA-protein assemblies including processing bodies (P-bodies) and stress granules (Malinovska et al. 2013; Protter and Parker 2016). Prion-like domains constitute as subclass of intrinsically disordered domains typically enriched in glutamine, asparagine, and aromatic residues. They are suggested to mediate multivalent protein-protein interactions allowing for self-assembly and protein phase separation, leading to the formation large, membrane-free assemblies. It is therefore tempting to speculate that Hsp42 aggregase activity originates from its prion-like domain; however, direct evidence is missing so far.

The aggregase function of Hsp42 seems to include two distinct activities. First, Hsp42 directly promotes protein aggregation as fluorescent thermolabile reporters form foci during stress conditions in yeast wild type but not in *hsp42*Δ cells. Second, Hsp42 mediates the coalescence of multiple small CytoQs into fewer CytoQs of enlarged size. By promoting protein aggregation, Hsp42 counteracts the activity of Hsp104, the yeast disaggregase of the Hsp100 family. These counteracting activities become obvious when studying cytosolic aggregation of fluorescent reporter proteins in *hsp42*Δ *hsp104*Δ double knockout cells. Here, partial aggregation of the reporters can be observed, in contrast to *hsp42*Δ cells. The dependence on Hsp42 for protein aggregation can thus be at least partially overcome by inhibiting aggregate solubilization (Escusa-Toret et al. 2013; Saarikangas and Barral 2015). The aggregate coalescence activity of Hsp42 was demonstrated by re-addition of Hsp42 to *hsp42*Δ *hsp104*Δ cells harboring multiple small aggregates, causing aggregate fusion to a single focus (Saarikangas and Barral 2015). It is currently unknown whether the two observed Hsp42 activities (promoting protein aggregation, mediating coalescence of small aggregates) are mechanistically distinct, eventually relying on different Hsp42 domains or involving cooperation with distinct cellular factors.

The mechanistic details of Hsp42 aggregase activity remain poorly defined. An Hsp42 aggregase activity could be recently reconstituted in vitro using thermolabile MDH as model (Ungelenk et al. 2016). Upon temperature upshift to mild denaturing conditions for MDH (41 °C, which is well below its  $T_M$  of 51 °C), the presence of Hsp42 is critical to promote the formation of light-scattering aggregates/assemblies. These conditions likely cause only partial MDH unfolding which effectively lowers the concentration of unfolded molecules and exposed hydrophobic patches in the substrate as compared to that of unfolding at temperatures around the  $T_M$ . In consequence, MDH on its own is not forming light-scattering aggregates within the time frame of the experiment, whereas the presence of Hsp42 even at substoichiometric levels triggers MDH aggregation. Hsp42 does not accelerate MDH unfolding, excluding that it acts by

**Table 1** Hsp42-dependent protein aggregation. Protein substrates that are deposited at CytoQs and chaperones (Ssa1, Hsp104) reporting on CytoQ formed by endogenous yeast proteins are listed. The stress regimes or genetic backgrounds inducing protein aggregation and respective references are given

Substrate/endogenous aggregate marker	Stress condition/genetic background	Reference
VHL, Ubc9 <sup>ts</sup> , luciferase, Hsp104	heat shock +/- proteasome inhibition (+MG132, cim3-1 <sup>ts</sup> )	(Escusa-Toret et al. 2013; Miller et al. 2015a; Song et al. 2014; Specht et al. 2011)
GFP-DegAB	<i>ssa1</i> Δ <i>ssa2</i> Δ	(Shiber et al. 2013)
tGnd1	<i>ubr1</i> Δ <i>san1</i> Δ	(Miller et al. 2015a)
Inactivated 26S proteasome, dysfunctional 26S proteasome Ssa1, Hsp104	Proteasome inhibition (MG132), <i>doa5-1</i> , <i>rpn5</i> ΔC Aging	(Marshall et al. 2016; Peters et al. 2015) (Saarikangas and Barral 2015)

increasing the concentration of hydrophobic patches in the substrate. Hsp42 might instead nucleate mixed complexes by promoting MDH-Hsp42 interactions with higher on-rates than those of MDH-MDH interactions during aggregation (Ungelenk et al. 2016). The reconstitution of Hsp42 aggregase activity *in vitro* shows that this sHsp is necessary and sufficient to promote protein aggregation. Additional cellular factors for CytoQ formation are therefore not required and indeed have not yet been reported.

#### Refolding vs. degradation: destiny of substrates sequestered in CytoQ deposits

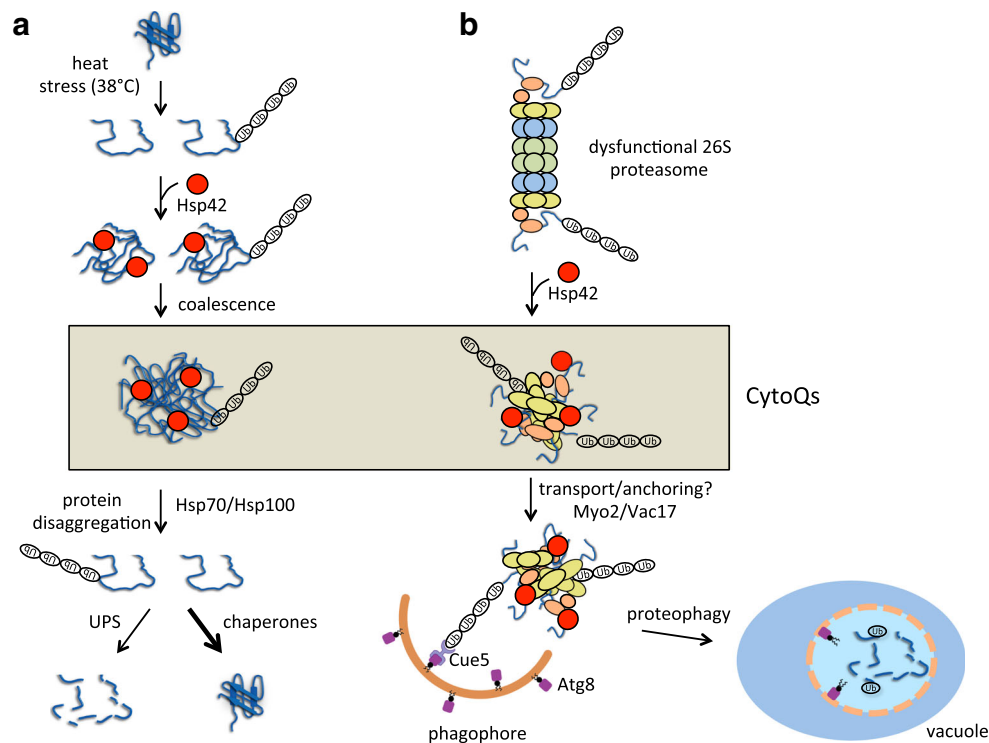
What is the fate of substrates deposited at CytoQs? There is no simple answer to this question and the outcome will depend on the particular substrate and applied stress condition. Heat stress-induced CytoQs are efficiently solubilized during stress recovery period by action of the Hsp70/Hsp100 chaperone system (Fig. 2) (Miller et al. 2015a; Specht et al. 2011). In this sense, CytoQs represent classical protein aggregates. Any triage of the sequestered substrates between degradation and refolding pathways will therefore be made only after dissociation from CytoQ and will depend on refolding kinetics of the substrate and its affinity for chaperones and proteases (Fig. 2). Heat stress-induced CytoQs thus do not contain any fate-decision information and do not appear to selectively target substrates to refolding or degrading pathways.

In contrast to these generic features valid for bulk misfolded proteins sequestered in CytoQs, a specific role of Hsp42 and CytoQs was recently reported for dysfunctional and inactive proteasomes, which cannot assemble properly because of mutations or inhibitions (Marshall et al. 2016; Peters et al. 2015). While misassembled proteasomes are preferentially degraded by the ubiquitin-proteasome system involving 26S wild type proteasomes, they become substrate of Hsp42 if stabilized upon proteasome inhibition and are deposited at CytoQs. Accumulation of misfolded proteins, which would otherwise be degraded by intact or active 26S proteasomes, might additionally contribute to the aggregation

process. Notably, CytoQ formation is a prerequisite for subsequent clearance of the sequestered proteasomal subunits by a specific form of selective autophagy, proteophagy (Marshall et al. 2016) (Fig. 2). Autophagy involves the formation of a double-layered membrane structure that engulfs cellular content followed by fusion with the vacuole, leading to the degradation of enclosed components by vacuolar peptidases. Whereas bulk autophagy non-selectively targets cytosolic content, selective autophagy targets specific substrates. Interestingly, Hsp42-dependent selective autophagy of dysfunctional proteasomes involves Cue5, which was previously linked to aggregophagy of polyQ protein aggregates (Lu et al. 2014). Cue5 acts as an adaptor protein linking ubiquitinated proteins to the autophagy receptor Atg8, present at autophagosomal membranes (Lu et al. 2014; Marshall et al. 2016). The crucial role of CytoQ as intermediate compartment for autophagic clearance of special substrates could be explained by its reported position next to the vacuole (Marshall et al. 2016; Spokoini et al. 2012). This location might enable Cue5 to deliver the sequestered substrates to the nearby Atg8 receptor lining the phagophore (Fig. 2).

Whether ubiquitination of the defective proteasomes is happening before or after CytoQ formation is not known. Ubiquitination was originally described as sorting signal for CytoQ formation (Shiber et al. 2013); however, Hsp42-dependent protein sequestration also happens in the absence of substrate ubiquitination (Miller et al. 2015a). It therefore seems unlikely that CytoQ formation is needed for substrate ubiquitination or, vice versa, that ubiquitination functions as obligatory CytoQ sorting signal.

The navigation of a specific subset of CytoQs, including defective proteasome particles, to a specific site at the vacuole demands for a specific transport route. Notably, Vac17, a protein linked to vacuolar inheritance, has been recently linked to CytoQ formation (Hill et al. 2016). Vac17 functions as a vacuole-specific receptor for the Myo2 motor protein that is involved in actin-based transport of cargo. Vac17 overproduction causes aggregate condensation, whereas *vac17*Δ cells exhibit an increased number of cytosolic aggregates (Hill



**Fig. 2** Destiny of substrates sequestered at CytoQs in an Hsp42-dependent process. **a** Formation of cytosolic aggregates (CytoQs) under physiological heat stress (38 °C) requires Hsp42, which promotes protein aggregation and mediates aggregate coalescence. Substrates deposited at CytoQs are rescued by the Hsp70-Hsp100 disaggregase system. Solubilized substrates are either refolded or degraded by the ubiquitin-proteasome system (UPS). **b** Dysfunctional, ubiquitinated 26S

proteasomes are sequestered in an Hsp42-dependent manner. CytoQs including dysfunctional 26S proteasome subunits are potentially transported and anchored to the vacuole in a Myo2- and Vac17-dependent process. The adaptor protein Cue5 links the sequestered proteasomal subunits to the Atg8 receptor at the phagophore. This results in the formation of autophagosomes and the engulfment of CytoQs, which are finally degraded by vacuolar peptidases

et al. 2016). The number of heat stress-induced CytoQs is also increased when affecting Myo2, the actin cytoskeleton, or vesicle trafficking pathways (Song et al. 2014). It is therefore suggested that CytoQs are tethered to actin cables for Myo2-dependent transport (Song et al. 2014). Which factors might link Hsp42-containing CytoQs to actin cables are unknown. A direct, essential involvement of Hsp42 seems unlikely as affecting actin-based vesicle transport also increases the numbers of amyloid inclusions formed by e.g., Htt103Q-GFP or NM-GFP whose aggregation is Hsp42-independent (Kumar et al. 2016; Song et al. 2014).

### Outlook: do sHsps generally function as aggregases?

How widespread is the function of sHsps as cellular aggregases? Experimental evidence supporting an aggregase activity of other sHsps has been reported. Overproduction of IbpA/IbpB in *E. coli* increases inclusion body formation by recombinant proteins (Han et al. 2004). In *Schizosaccharomyces pombe*, the sHsp Hsp16 is involved in protein-aggregate coalescence, thereby affecting aggregate segregation and increasing stress sensitivity (Coelho et al. 2014). Human HspB7 associates via its N-terminal region with nuclear SC35 splicing speckles and the Cajal bodies

(Vos et al. 2009). Long-lived *C. elegans daf2* mutants show an increased aggregate load compared to age-matched control animals. Increased protein aggregation correlates with an increased abundance of specific sHsps in the insoluble cell fraction, implying that sHsps might take part in a cytoprotective aggregation response during aging (Walther et al. 2015). Notably, not only the extent of protein aggregation is changed in *C. elegans daf2* mutants but also the nature of aggregated proteins (Walther et al. 2015). sHsps might therefore not only control the degree but also the specificity of the aggregation process.

However, an Hsp42 homolog, representing the best-studied prototype of a cellular aggregase, does not exist in bacteria, plants, or metazoans. Most organisms do not encode for sHsps harboring a putative prion-like domain. The absence of a direct Hsp42 homolog does however not exclude an aggregase function of other sHsps for two reasons. First, the NTEs of sHsps are generally structurally disordered and many NTEs are enriched in aromatic residues as observed in prion-like domains (Kriehuber et al. 2010). sHsps thereby might exhibit aggregase activity via their NTEs, while these NTEs are not recognized as prion-like domains in bioinformatic surveys. Second, we shall not strictly separate between sHsps exhibiting holdase and aggregase activity. We rather suggest

that the two, so far separately described, sHsp functions represent the two outer borders of a continuum of differently sized sHsp/substrate complexes. The size of sHsp/substrate complexes will depend on various parameters, including specific sHsp features, the sHsp-substrate ratio, the substrate identity, and the applied stress regimes. sHsps might differ in the degree of aggregase activity, defined by sHsp/substrate complex size and solubility, but not by their ability to actively sequester substrate proteins.

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