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Using Single-Molecule Approaches to Understand the Molecular Mechanisms of Heat-Shock Protein Chaperone Function

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Abstract

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Using Single-Molecule Approaches to Understand the Molecular Mechanisms of Heat-Shock Protein Chaperone Function

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Abstract

The heat-shock proteins (Hsp) are a family of molecular chaperones, which collectively form a network that is critical for the maintenance of protein homeostasis. Traditional ensemble-based measurements have provided a wealth of knowledge on the function of individual Hsps and the Hsp network; however, such techniques are limited in their ability to resolve the heterogeneous, dynamic and transient interactions that molecular chaperones make with their client proteins. Single-molecule techniques have emerged as a powerful tool to study dynamic biological systems, as they enable rare and transient populations to be identified that would usually be masked in ensemble measurements. Thus, single-molecule techniques are particularly amenable for the study of Hsps and have begun to be used to reveal novel mechanistic details of their function. In this review, we discuss the current understanding of the chaperone action of Hsps and how gaps in the field can be addressed using single-molecule methods. Specifically, this review focuses on the ATP-independent small Hsps and the broader Hsp network and describes how these dynamic systems are amenable to single-molecule techniques.

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Introduction

Most proteins need to fold into their three-dimensional native conformation in order to carry out their biological function. Instead of fast and efficient folding into a functional state, many proteins undergo kinetic partitioning between various denatured, intermediate and native ensembles throughout their lifetime. During periods of cellular stress (e.g., changes in temperature, pH, redox state) or as a result of certain genetic mutations, the ability of proteins to fold is disrupted and the formation of intermediate and misfolded species becomes favored. High concentrations of misfolded species can lead to protein aggregation, which has been implicated in numerous diseases ranging from cataract [1] to Parkinson's disease [2]. Consequently, cells have evolved a complex network of mechanisms that act to prevent protein aggregation and maintain protein homeostasis (proteostasis) [3].

Molecular chaperones play a vital role in the proteostasis network by facilitating protein folding or degradation, thereby preventing protein aggregation [4,5]. In humans, there are 332 genes that encode for the chaperone and co-chaperone families, together making up the “chaperome” [6]. A large subset (but not all) of these chaperone genes encode for the heat-shock protein (Hsp) family of molecular chaperones, which were originally identified due to their dramatic up-regulation under conditions of cellular stress. This highly conserved group of molecular chaperones is divided into several classes according to their monomeric molecular mass, that is, Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small Hsps (sHsps) [7]. Traditionally, these Hsps have been referred to as either ATP-dependent or ATP-independent chaperones. The ATP-dependent chaperones, which include the Hsp70, Hsp60, Hsp90 and Hsp100 classes, utilize repeated cycles of ATP hydrolysis to actively assist in folding or

unfolding of client proteins. Conversely, ATP-independent chaperones, such as the sHsps, do not actively refold or unfold clients but instead recognize and bind aggregation-prone proteins to stabilize them and prevent their aggregation [5]. Recently, there has been a growing awareness that some Hsps can interact with proteins that are already aggregated, rather than just aggregation-prone monomeric forms [8–11].

Hsps have been well studied and it is commonly accepted that these chaperones, in conjunction with co-chaperones, interact and work together as a complex, multi-component network to promote correct folding of nascent and misfolded proteins (further reviewed in Refs. [12]). The extent and complexity of the interactions between Hsps and their co-chaperones is highlighted by a STRING network analysis (Fig. 1), which emphasizes the importance of the Hsp70 chaperones as central components within this network. Much of the focus

regarding the cooperative ability of Hsp classes has been on the interplay between ATP-dependent Hsps [14,15]. However, our understanding of how client proteins are shuttled between the different components of the Hsp machineries is still not well understood. In addition, there is a need for a deeper understanding of the role that the ATP-independent sHsps play in the larger Hsp network. One of the difficulties in studying the Hsp network (and the functions of individual Hsps within it) is the dynamic nature of the interactions they make with each other and their client proteins. This is particularly the case for many of the eukaryotic sHsp homologues, which have a propensity to form dynamic, polydisperse oligomers in solution [16,17]. Consequently, rare and transient interactions between chaperones and their clients are difficult to observe using traditional solution-phase, ensemble-averaging techniques. However, this limitation can be overcome in single-molecule experiments as individual protein

Fig. 1. STRING network analysis of the Hsp interactome. Proteins from the Hsp families; Hsp40, Hsp70, Hsp60, Hsp90, Hsp100 and sHsps along with some of their co-chaperones were entered into the STRING database (<https://string-db.org>) [13]. Color of nodes denotes the family of chaperones: Hsp40 (blue), Hsp70 (orange), Hsp60 (pink), Hsp90 (green), Hsp100 (purple), sHsps (red) and co-chaperones (yellow). The confidence level was set to 0.400 (medium) and the line thickness indicates the level of data support (based on experiments, gene fusions and databases).

interactions can now be observed in real time [18]. As such, the kinetic insight provided by these methods makes them ideally suited to the study of Hsp function.

The suitability of single-molecule techniques to study complex, multi-component cellular machineries has previously been demonstrated in the fields of DNA replication and motor proteins (reviewed in Ref. [19]). These methods have also been applied for the study of chaperones as protein folding machines, with the number of Hsp single-molecule studies rapidly increasing since the first report published more than 20 years ago [20]. These studies have traditionally utilized a combination of fluorescence-based methods and force spectroscopy to gain mechanistic insights into the dynamic activity of Hsps. Both fluorescence and force techniques can be used to probe different, but complementary, questions regarding chaperone dynamics, function and how these influence the conformation of the client protein. The most commonly used force spectroscopy techniques are atomic force microscopy and optical tweezers, both of which involve the controlled stretching and relaxation of polypeptides to enable the observation of rare and transient intermediate states that a protein occupies during unfolding or refolding [21]. Single-molecule fluorescence techniques rely on the use of fluorophores to allow visualization of individual proteins via imaging-based techniques such as total internal reflection fluorescence (TIRF) and confocal microscopy. Fluorescence-based experiments are particularly amenable for the direct temporal observation of protein conformational dynamics, protein–protein interactions and for measuring the kinetics of client binding by chaperones [22].

The first part of this review provides an overview of the current mechanisms by which the different classes of Hsps are thought to interact with client proteins, with a particular focus on the molecular action of sHsps and the cooperative aspects of Hsp networks. We then discuss how single-molecule techniques have been used to address questions in the Hsp field that have been difficult to study using ensemble measurements. The review concludes by highlighting how single-molecule techniques can be used to better understand the molecular mechanisms by which sHsps and the broader Hsp network function to keep the proteome folded and functional.

ATP-dependent Hsps

In the past few decades, there has been a significant amount of research into ATP-dependent chaperone function, due to their critical importance in the folding of polypeptides and their role in numerous other cellular processes [23]. As these chaperones utilize the energy from ATP hydrolysis to help

actively fold their clients to the native state, they are often referred to as “foldase” chaperones. The individual functions of the above-mentioned ATP-dependent chaperones are briefly discussed below.

The Hsp70/Hsp40 system, a central hub of the Hsp network

Hsp70 (DnaK in bacteria), in conjunction with its co-chaperone Hsp40 (DnaJ in bacteria), mediates the initial stages of protein folding and acts as a central hub in the chaperone network within the cell [24]. Hsp70 consists of a N-terminal nucleotide-binding domain (NBD) and a C-terminal substrate-binding domain (SBD), which itself is composed of a β -sandwich peptide-binding subdomain and a α -helical lid [25]. In the ATP-bound state, the α -helical subdomain of the SBD is docked onto the NBD, resulting in an “open” conformation that is characterized by high on/off rates and low client affinity (Fig. 2A). Binding of unfolded, partially folded or misfolded client proteins by Hsp70 can occur independently or via Hsp40-mediated recruitment, whereby interactions with hydrophobic sequences from the client protein are established in the β -sandwich subdomain of Hsp70 [29–31]. Hydrolysis of ATP to ADP, which is accelerated >1000 fold in the presence of Hsp40 [32,33], results in domain undocking and closing of the α -helical lid over the β -sandwich subdomain to trap the bound client (resulting in low on/off rates and high client affinity). Release of ADP from the NBD, catalyzed by nucleotide-exchange factors (NEFs; e.g., GrpE in prokaryotes, Bag1 and others in eukaryotes), mediates release of the client protein and returns Hsp70 to the open conformation. Repeated cycles of client binding and release, driven by nucleotide exchange, are thought to direct the client down efficient folding pathways to the native state.

Hsp60, a confining cage for protein folding

Some proteins can only be partially folded by the Hsp70/40 network of chaperones and therefore require additional assistance from the Hsp60 family of chaperones (also termed chaperonins) in order to acquire a folded functional conformation [29]. Eukaryotes contain the Tric/CCT homologue of Hsp60, while the corresponding homologue in bacteria (GroEL/GroES) is the simplest and most studied Hsp60 system [34]. GroEL/GroES has a double-ring structure (*cis* and *trans* rings) comprising many closely related subunits. These each consist of a client-binding apical domain, an intermediate domain and an ATP-binding domain [35]. When GroEL is in the open-state, exposed hydrophobic residues from the client-binding apical domain facilitate recognition and binding of misfolded proteins (Fig. 2B). Binding of multiple ATP molecules to the client-bound ring

Fig. 2. Reaction cycle of different ATP-dependent chaperones. (A) The Hsp70 reaction cycle. (1) Hsp40-mediated delivery or Hsp70-mediated capture of client proteins (2) Hsp40-mediated hydrolysis of Hsp70-ATP yields the Hsp70-ADP high client-affinity state. (3) NEF promotes release of ADP and the client, which can either refold to the native state or be recaptured by Hsp70. Rebinding of ATP returns Hsp70 to the “open” conformation for the start of the next reaction cycle. (B) Folding cycle of GroEL/GroES. (1) Misfolded proteins can bind to the open *trans* ring of GroEL independently or via a DnaK/DnaJ-mediated mechanism. (2) Binding of ATP and GroES to the client-bound ring releases the bound client into the GroEL cavity to form a new *cis*-ternary complex. During this step, conformational changes occur that allow GroEL to accommodate proteins up to 60 kDa for folding. (3) Protein folding occurs during the period that it takes for the ATP to be hydrolyzed on the *cis* ring (~10 s). (4) Binding of ATP to the *trans* ring causes the dissociation of ADP and the GroES subunit to release the native protein and restores GroEL to the original *cis*-ternary complex. Note that under this reaction cycle depicts GroEL as an asynchronous structure in which the folding of clients alternates between the two rings; however, synchronous folding in both rings has also been reported [26–28]. (C) Folding cycle of Hsp90. (1) The apo-Hsp90 state is formed following ADP release from ADP-Hsp90. Clients are then delivered by Hsp70/co-chaperone complexes to apo-Hsp90. (2) Binding of ATP to Hsp90 causes the slow dimerization of both NBD domains to trap the delivered client. (3) Close association of the NBDs and middle-domains causes Hsp90 to adopt a tight, twisted conformation. (4) ATP hydrolysis is mediated by specific residues from the middle-domain that results in the release of the now native client. (D) Dissagregase cycle of Hsp100. (1) Binding of ATP to Hsp100 assists in the binding of aggregated clients via a process that is assisted by Hsp70. (2 and 3) Repeated cycles of ATP hydrolysis and ADP release around the ATPase rings of Hsp100 drive the translocation of the bound client through the central pore. (4) Eventually, the client is pulled through the pore and released into the cytosol for refolding. Release of ADP returns Hsp100 to the beginning of the reaction cycle.

catalyzes the release and sequestration of the client into the central cavity of GroEL, where it becomes encapsulated following binding of the GroES co-chaperone “lid” to GroEL [34–36]. Binding of ATP and GroES results in significant structural rearrangements of GroEL that increases the size of the cavity, which can accommodate proteins up to 60 kDa, and positions hydrophilic residues inward [35,37]. Consequently, the captured client is sequestered into an environment that is conducive to folding without the additional risk of erroneous interactions with other proteins that could lead to aggregation [38]. Productive folding occurs until ATP is hydrolyzed, upon which ATP binding to the adjacent *trans* ring causes the dissociation of GroES and release of the client. Should the released protein be unable to acquire the correct fold, it has the opportunity to rebind GroEL and undergo repeated folding cycles until the native state is acquired. In the model of GroEL/GroES-assisted folding described above, the reaction cycle occurs in an asynchronous manner (i.e., one client is folded within a ring at any point in time). Recently, a synchronous model of GroEL/GroES-assisted folding has been observed in which a client protein can be processed in both rings simultaneously [26,27,39–41]. However, further investigation is required to better understand this mechanism of Hsp60 action. It is thought that the eukaryotic Hsp60 homologue, TriC/CCT, follows a similar reaction cycle as GroEL/GroES, although this process occurs at a slower rate and protein encapsulation relies on closure of an inbuilt lid segment located on the apical domain (in place of GroES) [42,43].

Hsp90, a ubiquitous Hsp with a select clientele

Some polypeptides require Hsp90 to mediate the final stages of protein folding so that they can acquire their biological function. For instance, Hsp90 plays a critical role in assisting the final assembly of some multi-protein complexes and the maturation of specific clients. Compared to other classes of Hsps, the binding of client proteins by Hsp90 is less promiscuous; instead, Hsp90 interacts with a select, yet structurally and functionally diverse clientele that ranges from nuclear hormone receptors and protein kinases to transcription factors [44,45]. Eukaryotes contain several Hsp90 isoforms that reside in different cellular compartments (e.g., the cytosol, mitochondria or endoplasmic reticulum) and therefore interact with different clients and co-chaperones [46]. Hsp90 is a homodimeric protein, each subunit consisting of a N-terminal NBD, a middle SBD and a C-terminal dimerization domain. Similar to Hsp70 and Hsp60, the cycle of ATP hydrolysis is accompanied by significant structural rearrangement of Hsp90 (Fig. 2C). In the nucleotide-free (apo) or ADP-bound state, Hsp90 exists in a V-shaped structure that dynamically samples open and closed conformations

[47]. Client proteins are recruited to apo-Hsp90 by the Hsp70 system and other co-chaperones [48,49]. Upon binding of ATP to Hsp90, a lid segment closes over the bound nucleotide and facilitates the dimerization of the two-nucleotide binding domains by exposing the dimerization interfaces, thus enabling Hsp90 to bind to the client protein [50,51]. Close association of the middle and NBDs results in twisting of each subunit around each other, with specific residues from the middle-domain contributing to ATP hydrolysis and release of the folded client [52]. At various stages throughout the Hsp90 cycle, numerous co-chaperones play a significant role in mediating ATP-hydrolysis reactions (i.e., speed up or slow down hydrolysis) and directing the specificity of the Hsp90 clientele by recruiting clients ([53], reviewed in Ref. [54]). More than 20 co-chaperones have been identified to interact with cytosolic Hsp90 and regulate its activity. However, the identification and precise role of co-chaperones to regulate the activity of other Hsp90 isoforms remains to be established [55].

Hsp100, the disaggregation machine

The Hsp100 family of molecular chaperones belongs to the AAA+ protein superfamily [56]. This family shares an AAA domain that is essential for nucleotide binding, hydrolysis and oligomerization, and is critical for their disaggregase function. The two most studied Hsp100 chaperones, ClpB (in bacteria) and Hsp104 (in yeast), are hexameric structures comprising individual subunits that contain an N-terminal domain and two oppositely oriented AAA domains (termed AAA-1 and AAA-2) separated by a coiled-coil middle-domain [57,58]. Unlike other Hsps that predominantly bind to monomeric clients, Hsp100 binds to aggregated forms of protein to mediate their solubilization. Aggregated clients are bound and recruited to Hsp100 by Hsp70, whereby the client is pulled and unfolded through a central pore within Hsp100 in a nucleotide-dependent manner (Fig. 2D). The binding and hydrolysis of ATP to Hsp100 is extremely complex and finely regulated by allosteric communications between neighboring AAA domains (either in the same or adjacent ATPase AAA-ring system). By examination of the simpler, single-ringed ClpX Hsp100 chaperone, it is thought that coordinated cycles of ATP hydrolysis enable more efficient and forceful client translocation through the ring [59]. However, the unfolding force exerted by Hsp100 may sometimes be insufficient to fully unfold highly stable, tight-binding domains within the client, resulting in partially folded structures being pulled through or partially threaded through the pore [60–62]. Clients that are unable to be properly processed may be released into the cytosol upon rapid dissociation of Hsp100 subunits, whereby the

dynamical nature of subunit exchange acts as a mechanism to prevent blocking of the Hsp100 pore by clients. However, subunit exchange and dynamics have also been observed to be a common process during its function as a disaggregase [62], with partial unfolding sufficient to solubilize aggregates [60]. Regardless of whether the client is released into the cytosol or completely pulled through the Hsp100 pore, the client is separated from the aggregate and can be refolded by other Hsp chaperones [63,64].

Outstanding questions regarding the mechanisms of ATP-dependent Hsps

While significant progress has been made to understand the role ATP-dependent chaperones play in maintaining proteostasis, the precise molecular mechanisms by which they act to refold proteins and prevent their aggregation remain elusive. For instance, it is not well understood precisely what role ATP hydrolysis has in Hsp70/Hsp40, Hsp60 and Hsp90-assisted folding. Do ATP-driven cycles of client binding and release stimulate folding? Or are they utilized to directly alter the conformational state of the client to generate productive folding intermediates? It is also uncertain how the conformational dynamics of the chaperone (e.g., movement of the α -helical subdomain on Hsp70) affects the conformation, entropy or folding pathways of bound client proteins. It is known that client proteins can undergo multiple cycles of binding and release from ATP-dependent chaperones [32,65,66]. However, it is not clear whether a single chaperone is involved throughout the entirety of this process or if there is dynamic exchange between multiple chaperone molecules that are within close proximity of the client. In some cases, such as with Hsp90 and Hsp100, the precise mechanisms by which domain allostery, co-chaperones and other regulators influence the ATP-hydrolysis cycle (and subsequently how they affect the conformation of the client) are also not well understood. While some studies have investigated the dynamics of Hsp100 subunit exchange [60–62], the precise kinetics of this process and its influence on the disaggregase and ATPase activity of Hsp100 remains unclear. Furthermore, the exact mechanism by which some of these ATP-dependent Hsps cooperate synergistically with ATP-independent chaperones, such as the sHsps, remains elusive.

ATP-independent chaperones—sHsps

The sHsps are a family of ubiquitous intracellular ATP-independent chaperones. There are 10 sHsp genes in humans (*HSPB1–10*) [67]; these show tissue-specific expression, with some being widely

expressed (e.g., Hsp27, HSPB1) and others showing very restricted tissue expression (e.g., HSPB9). In addition, the levels of some (but not all) sHsps in cells are dramatically increased under stress conditions. The sHsps are characterized by the presence of a highly conserved central α -crystallin domain (ACD), which is approximately 80 amino acids in length, which is flanked by less conserved N- and C-terminal regions that are of variable length between sHsp members [68]. These chaperones are classified as sHsps because their monomeric masses are relatively low (15–40 kDa) compared to other Hsps; however, their name is somewhat of a misnomer since many of them form large oligomeric species in solution [69]. The structural polydispersity of some sHsp oligomers is highlighted by α B-crystallin (HSPB5), which forms oligomers in solution ranging in mass between 420–980 kDa, which corresponds to between 24 and 33 monomers [70].

The oligomers formed by the large oligomeric forms of mammalian sHsps are dynamic in that they undergo rapid subunit exchange, not only with other subunits of the same type (homo-oligomerization) but also with other sHsps (hetero-oligomerization) [17]. There is conflicting evidence of the effect that subunit exchange has on the chaperone activity of sHsps, with some studies demonstrating that an increase in the rate of subunit exchange results in higher chaperone activity for α -crystallin [17]. However, other work has reported that a mutant form of α A-crystallin (α A-crystallin1-168, HSPB4), which displayed a significantly decreased rate of subunit exchange, was found to have chaperone efficiency similar to wild-type α A-crystallin [71]. Furthermore, when isolated ACDs of α B-crystallin are locked into a dimer form (via mutagenesis to create a disulfide-bond at the dimer interface), they are as active as ACDs capable of exchanging monomers [72]. Together, these findings suggest that chaperone activity of sHsps is not reliant on subunit exchange. However, further studies are required to provide definitive evidence to support such a model.

Despite the increasing number of studies that investigate the interactions between sHsps and misfolding proteins, the precise region or regions that mediate binding to client proteins remain to be identified. For example, multiple regions of Hsp27, essentially covering the entire protein sequence, have been suggested to act as client-binding regions [73–76]. Therefore, it is suggested that sHsps do not have a universal client protein-binding site, but instead exploit multiple hydrophobic sites on their surface to interact with a diverse range of proteins. The promiscuous binding of client proteins by sHsps is consistent with the finding that they interact with a plethora of proteins *in vitro* [77–80] and affect a wide range of cellular functions *in vivo* [81].

A recent consensus model of sHsp chaperone action describes interactions with client proteins

occurring through either (i) weak, transient interactions between sHsps and partially misfolded proteins resulting in the aggregation-prone protein being stabilized such that it can re-enter the protein “folding” pathway and thus refold to the native state, or through (ii) high-affinity stable interactions, forming stable high-molecular-mass complexes with destabilized protein intermediates at risk of hydrophobic collapse and aggregation (Fig. 3) [82]. Both types of interactions can occur with the same client protein, as demonstrated by the ability of α A-crystallin and α B-crystallin to undergo both transient and stable interactions with the proteins citrate synthase [83] or α -lactalbumin [84,85]. The factor that appears to govern the mode of binding is the extent of exposed hydrophobicity on the partially folded protein (and hence the rate of aggregation). Thus, it has been postulated that sHsps generally act through weak, transient interactions under normal cellular conditions and rely on higher-affinity client interactions (i.e., formation of sHsp–client complexes) during periods of cellular stress when proteins are more destabilized [85,86].

The consensus model postulates that uncomplexed, dissociated sHsps, generally depicted as a dimer, are the chaperone-active species that bind to aggregation-prone proteins to perform the initial “holdase” chaperone function (Fig. 3). Based on this model, the larger oligomeric species act as reservoirs for these smaller chaperone-active forms, the latter having increased exposure of hydrophobic regions and therefore higher affinity for misfolded proteins. Once the dissociated species binds to a client protein, the sHsp–client protein complex can be sequestered into a high-molecular-mass sHsp–client complex. From this complex, aggregation-prone client proteins can be either shuttled for degradation or held in a folding-competent state for subsequent refolding by ATP-dependent chaperones (e.g., the Hsp70 system) [82].

While this model accounts for much of our current knowledge on sHsps, there are a number of critical aspects of their function where definitive data are lacking to support the model described above. This hiatus in our understanding is predominantly due to the significant polydispersity and inherent heterogeneity

Fig. 3. A consensus model for the chaperone mechanism of sHsps. The sHsps are in a dynamic equilibrium between large and small oligomers. They are able to form weak interactions with relatively stable protein intermediates that have left the protein-folding pathway, enabling them to re-enter the folding pathway and therefore refold to their native conformation. If a partially folded intermediate leaves the folding pathway and is in a destabilized state, it can be recognized by dissociated sHsps, which bind to it and therefore stabilize the protein. This high-affinity interaction results in the formation of a sHsp–client complex that is able to re-associate with large sHsp oligomers to form a high-molecular-mass complex. Bound client proteins can then be either shuttled for degradation or held until they can be refolded back to their native conformation via the action of “foldase” chaperones. Diagram adapted from Treweek *et al.* [82].

that arises from rapid and dynamic exchange of sHsp subunits. Furthermore, the exact role sHsp subunit exchange, and the resulting changes in oligomeric size, has on the chaperone activity of sHsps remains enigmatic. For example, there is no definitive evidence that the large oligomers are not chaperone active and that dissociated sHsp species are the only form capable of recognizing and interacting with client proteins. In fact, for some sHsps, such as α B-crystallin, there is no evidence of discrete dissociated species (i.e., dimers) existing in solution. While sHsps are known to be capable of transiently stabilizing aggregation-prone proteins in order to facilitate their refolding, they can also prevent aggregation by formation of stable, high-affinity complexes; the underlying mechanism by which sHsps mediate these different roles is poorly understood. Furthermore, questions remain regarding the stoichiometries of sHsp–client protein complexes and the kinetic processes (i.e., on/off rates) involved in client binding. Likewise, the precise mechanistic details by which sHsp-bound clients are passed over to ATP-dependent Hsps for refolding [87–89] remains elusive.

Hsp chaperone networks

Cells have evolved a vast network of Hsps and protein degradation systems in order to maintain a functional proteome. Both ATP-dependent and ATP-independent chaperones cooperate synergistically to regulate protein folding and prevent protein aggregation. As the interactions described below demonstrate, the Hsp70 class of molecular chaperones acts as a central component of this chaperone network, functioning with and coordinating other classes of Hsps to facilitate the correct folding of nascent and misfolded proteins [24].

Critical to the action of Hsp70 as a central hub in the chaperone network is its ability to promiscuously interact with a broad range of client proteins and conformational states [90,91]. For instance, Hsp70 acts early on unfolded nascent chains that emerge from the ribosome (Fig. 4, step 1) [92] but can also recognize proteins at later stages of protein folding. The action of Hsp70 is highly regulated by Hsp40s and NEFs, which assist in the capture, binding and release of client proteins. The prokaryotic Hsp70 system has been the most studied as it contains only a single isoform of Hsp70 (DnaK), Hsp40 (DnaJ) and NEF (GrpE). In this system, DnaJ is able to recognize and bind client proteins in a “holdase-like” manner and recruit them to DnaK for folding [93]. DnaJ then indirectly promotes DnaK-mediated protein folding by accelerating ATP hydrolysis, which facilitates binding of the client to DnaK and drives the reaction cycle forward [32]. The interaction between DnaK and DnaJ has been structurally characterized [94]; however, the transient nature of these interactions has made it difficult to determine

when DnaJ dissociates from DnaK during its folding cycle. In contrast to the prokaryotic Hsp70 system, eukaryotes have a significantly more complex Hsp70 system due to a higher number of components. For example, there are 13 isoforms of Hsp70, 50 Hsp40s and 7 NEFs in humans [7]. Furthermore, the Hsp70s of higher-order eukaryotes also have a plethora of co-chaperones, for example, HOP and CHIP, which assist in directing Hsp70 to other cellular pathways such as the Hsp90 or proteasome systems. Consequently, due to the complexity of the Hsp70 system in eukaryotes, it is significantly more difficult to study both *in vitro* and *in vivo*.

Proteins that are unable to be folded by the Hsp70 system can be transferred to the Hsp90 system to acquire their native fold (Fig. 4, step 2). While Hsp70-mediated delivery of clients to Hsp90 in prokaryotes occurs directly [95], in eukaryotes, the co-chaperone HOP is required to regulate interactions and client transfer between Hsp70 and Hsp90 [49,96,97]. HOP facilitates these interactions via its two-tetratricopeptide repeat (TPR) domains, which bind to the extended –COOH domains located on Hsp70 and Hsp90 [98]. The cooperation of this system is exemplified in the folding of signal-transduction proteins [99], whereby newly synthesized receptors are bound by Hsc70 (the constitutively expressed Hsp70) and transferred to Hsp90 via HOP for maturation of the folded receptor complexes [100]. In addition, the Hsc70/Hsp90 system also works in conjunction with the ubiquitin–proteasome system, via the co-chaperone CHIP, to degrade misfolded proteins [101]. CHIP is an E3 ubiquitin ligase that contains an N-terminal TPR domain that associates with Hsp90 or Hsp70 (the latter in conjunction with the Hsp70 cofactor Bag1) and targets its clients to the proteasome [102–105]. In conjunction with the Hsp70 system of chaperones, the activity and correct function of Hsp90 is also heavily regulated by a multitude of other co-chaperones (e.g., Aha1) that perform roles that include directing client specificity, ATP hydrolysis and transferring the client to other quality control systems.

In accordance with the role of Hsp70 as a central player in proteostasis, Hsp70 can also interact with newly synthesized polypeptide chains and maintain them in an unfolded state for transfer to the Hsp60 system for further folding assistance (Fig. 4, step 3). In *Escherichia coli* (*E. coli*), GroEL interacts with approximately 10% of all cytosolic proteins downstream of DnaK/DnaJ [106,107]. The ability of the Hsp70/Hsp60 chaperone network to fold a range of client proteins to a functional state has been previously demonstrated [24,92,108]. For example, sequential transfer of denatured rhodanese from DnaK/DnaJ to GroEL/GroES facilitates refolding and restoration of 70% of the rhodanese enzymatic activity [29]. The cooperative nature of the Hsp70/Hsp60 network is further exemplified by the overlapping of the DnaK interactome with many GroEL

Fig. 4. Schematic of the Hsp chaperone network. Hsp70 mediates the initial stages of protein folding and acts as a central hub in the Hsp chaperone network. Under physiological conditions, (1) Hsp70 in conjunction with its co-chaperone Hsp40 is able to bind unfolded nascent chains as they emerge from the ribosomes and actively fold them into their native conformation. If Hsp70/40 is only able to partially fold the protein, it can be transferred to either the Hsp90 (2) or Hsp60 (3) systems in order to acquire a folded functional conformation. Cellular stress conditions can cause proteins to misfold and associate into insoluble, toxic aggregates. (4) sHsps are able to recognize misfolded proteins and prevent their aggregation by sequestering them into a high-molecular-mass complex and subsequently transferring them to the Hsp70/40 system for refolding. (5) Hsp70/40 can associate with insoluble aggregates and mediate their transfer to the Hsp100 system for solubilization. The resulting unfolded proteins can then be transferred and refolded by the Hsp70/40 system.

clients (~30% DnaK clients interact with GroEL) and the finding that GroEL clients can accumulate on DnaK following GroEL depletion [24]. DnaK and GroEL are also complementary in that they recognize different conformational states of proteins. For instance, experiments with model clients have shown that DnaK has a preference for unstructured polypeptide chains, whereas GroEL binds with higher affinity to molten globule-like structures [29]. Consequently, this allows these two chaperone classes to efficiently cooperate and synergistically assist the folding of a wide range of clients.

During periods of cellular stress, sHsps can prevent protein aggregation by sequestering aggregation-

prone clients into high-molecular-mass complexes for subsequent transfer and refolding by the Hsp70 system (Fig. 4, step 4). This process has previously been demonstrated for a wide range of sHsps and their clients across both prokaryotic and eukaryotic systems [88,109–111]. For example, treatment of high-molecular-mass complexes consisting of luciferase or citrate synthase bound to α B-crystallin with purified Hsp70/Hsp40 results in the reactivation of the enzymes [112]. In addition, unfolded intermediates of citrate synthase that were bound to Hsp25 (the mouse ortholog of human Hsp27) could also be refolded by Hsp70 [109]. A recent study has illuminated how the sHsp–client complexes are structured such that

Hsp70 is able to efficiently bind and refold client proteins [111]. This study suggests that sHsp–client complexes consist of an immobile core with a dynamic sHsp shell, which prevents the access of proteases to the bound clients and allows for association of Hsp70 to the inner core of the complex for client refolding [111]. It has also been shown that client proteins that have been sequestered into high-molecular-mass complexes with sHsps are more efficiently disaggregated by the Hsp70 system (in conjunction with the Hsp100 disaggregation machinery) compared to aggregates in the absence of sHsps *in vitro* [113–115] and *in vivo* [116,117]. Based on these observations, it is proposed that during periods of cellular stress, sHsps sequester aggregation-prone clients in a folding-competent state that can be transferred to Hsp70 and/or Hsp100 for refolding upon return to physiological conditions.

If the Hsp network is overwhelmed with non-native species, these proteins can misfold and associate into insoluble, toxic aggregates. Emerging evidence has shown that some Hsps (e.g., Hsp70, Hsp100 and sHsps) can bind to these aggregated proteins [10,11,118] to prevent or minimize the cytotoxicity of these aggregates by mediating their solubilization or proteolysis [9,63,64,119,120]. *In vitro* studies have shown that ClpB (the Hsp100 homologue in *E. coli*) and Hsp104 (yeast homologue) are not able to efficiently disaggregate proteins alone, but require Hsp70 for its disaggregase activity [63,119] (Fig. 4, step 5). ClpB/Hsp104 exists in a repressed state in the absence of Hsp70 [121], which acts as a regulatory mechanism to prevent the unfolding and translocation of non-aggregated native clients in the cytosol [122,123]. Thus, ClpB/Hsp104 becomes highly specific toward aggregated clients due to the high local concentration of Hsp70 that accumulates on the surface of aggregates. The high density of Hsp70 molecules acts to promote recruitment, binding and activation of ClpB/Hsp104 in a process that requires two or more Hsp70s [123]. Alternatively, a recent study has suggested that ClpB recognizes hydrophobic regions of aggregated proteins and in doing so displaces Hsp70 from the surface of the aggregate by pulling the client through the central pore [124]. In this model, ClpB undergoes conformational changes that prevent further association with Hsp70 [125]. Clients can then be either fully or partially threaded through ClpB [60–62] for subsequent refolding in the cytosol.

The importance of single-molecule approaches for the study of Hsp chaperone activity

Traditionally, the structure and chaperone function of Hsps has been studied using ensemble-based techniques, which mask rare and transient species that may be present due to the averaging of billions of

asynchronous molecules. The established model of protein folding, whereby an ensemble of dynamic non-native structures is guided toward the most thermodynamically stable state, inherently implies that transient populations of rare folding intermediates are present during folding and there is significant heterogeneity in these species [126,127]. The complexity of this system is further enhanced by the involvement of chaperones such as the Hsps, due to the multiplicity of potential chaperone/co-chaperone interactions [18]. Consequently, the ability to decipher the molecular mechanisms by which chaperones assist protein folding and prevent aggregation is limited using ensemble measurement techniques. Recently, there has been significant interest in employing single-molecule approaches to study the mechanisms of chaperone function as they eliminate ensemble averaging through direct observation of a single-protein trajectory in real time, allowing detection of kinetic features and states that are normally hidden [128]. Moreover, single-molecule experiments do not rely on the synchronization of an ensemble of molecules, meaning that rare, transient and intermediate events of a single molecule can be viewed and measured, and its dynamics analyzed. For instance, by fluorescently labeling the light chain of myosin V and correlating the nanometer movements of fluorescence, the length of the transient intermediate “steps” has been determined to confirm the hand-over-hand mechanism utilized by myosin V to travel along actin filaments [129]. Furthermore, single-molecule experiments are especially amenable for characterizing the association and dissociation kinetics of bimolecular reactions [22], to the degree that the effect an individual parameter (e.g., on/off rates) has on the overall kinetics of a reaction can be determined [130].

Single-molecule techniques are particularly powerful for the study of chaperones, due to their capacity to follow the dynamic cycling between high and low client-affinity states in response to the presence of co-chaperones, nucleotides, temperature, client concentration and client conformation. To date, a combination of fluorescence and force spectroscopy techniques has been developed and applied to study protein folding and Hsp function at single-molecule resolution. Fluorescence-based experiments in particular are gaining popularity, as they are more amenable for the direct observation of chaperone-client interactions, changes in conformation as a client folds and measuring the kinetic rates and binding affinities of chaperones to clients. Studies that have used single-molecule techniques to provide new insights into the function and dynamics of Hsps are discussed below.

Hsps studied by single-molecule techniques

Recently, a combination of force and fluorescence-based single-molecule techniques has been used to

examine the chaperone activities of Hsps, with a particular focus on the dynamics and mechanistic aspects of ATP-dependent chaperones. Single-molecule studies have predominantly been used to examine the conformational dynamics of individual Hsps or their clients, revealing novel insights into Hsp function.

The Hsp70 system

A recent study by Kundel *et al.* [10] investigated the binding kinetics and stoichiometry of complexes formed between Hsp70 and the Alzheimer's disease-associated tau protein using a combination of TIRF microscopy and single-molecule fluorescence resonance energy transfer (smFRET). Briefly, during

TIRF microscopy, the excitation laser is totally internally reflected at the coverslip interface, which produces an evanescent field that penetrates the sample. The exponential decay of the evanescent field limits illumination and subsequent fluorophore excitation to ~150 nm into the sample from the coverslip surface, effectively eliminating signal from out-of-focus regions and dramatically enhancing signal-to-noise to enable observation of single molecules [131]. Using TIRF microscopy, the authors observed co-localization of Hsp70 with oligomeric and fibrillar forms of tau, but not monomeric species (Fig. 5A). Further analysis of the fluorescence intensity from co-localized Hsp70 and oligomeric tau species revealed that, at the molar ratios used in the TIRF experiment, the stoichiometry of binding was

Fig. 5. Stoichiometry and binding affinity of Hsp70 to tau at different stages of aggregation. (A) Direct visualization of the association between Hsp70 and monomeric, oligomeric and fibrillar forms of tau using TIRF microscopy. Hsp70 (blue) did not co-localize with monomeric tau (green, left panel), but did so when incubated with oligomeric (green and red) and fibrillar (yellow) tau species (middle and right panels respectively, red arrows). The fluorescence intensity of the co-localized species was analyzed to derive Hsp70: tau stoichiometries. (B) Saturation binding curves of Hsp70 to different forms of aggregated tau as assessed by smFRET and confocal microscopy. Hsp70 was observed to bind with higher affinity to larger tau species and K_D values (C) of Hsp70 to different species of aggregated tau could be measured. Figure taken with permission from Kundel *et al.* [10].

one Hsp70 to two tau monomers within each tau oligomer. To probe these interactions further, an smFRET system involving Hsp70 and monomeric tau was developed that allowed the binding affinities of Hsp70 to tau species at different stages of aggregation (i.e., from oligomers to fibrils) to be determined (Fig. 5B and C). Thus, K_D values could be extracted that would typically be inaccessible by conventional bulk measurements. From these experiments, it was observed that the binding affinity of Hsp70 to tau oligomers increased as a function of oligomer size, with the highest affinity ($K_D \sim 20$ nM) for tau fibrils. This presumably occurs as a result of a higher abundance of possible interactions sites for Hsp70 along the fibrils. This study concluded by suggesting that Hsp70 inhibits the nucleation and elongation of tau fibrils by binding to oligomers and smaller fibrils formed early during the aggregation process. In this way, binding of Hsp70 to oligomeric tau can reduce the toxicity associated with tau fibrillization in a manner similar to what has been observed for binding of sHsps to α -synuclein fibrils [11]. Thus, the binding of chaperones to early or fibrillar structures appears to be a general mechanism by which they mitigate the toxicity associated with these disease-related processes.

The ability of Hsp70 to recognize and stably bind different protein conformations described in Kundel *et al.* is consistent with other single-molecule experiments that describe the functional plasticity of Hsp70 [91]. Using optical tweezers, Mashaghi *et al.* found that DnaK bound to and stabilized near-native conformations of maltose binding protein (MBP), but not the natively folded protein, preventing its unfolding during subsequent pulling and relaxation cycles. Under high forces, MBP exists in a stretched linear-like conformation and folds to a compact, near-native conformation following a return to zero forces. However, when linearly stretched MBP was incubated in the presence of DnaK and then returned to zero forces, refolding was not observed. Instead, MBP remained in a globally unfolded state, presumably due to binding of DnaK. This observation is in agreement with previous single-molecule studies that have also reported DnaK binding to globally unfolded peptides [132,133]. Interestingly, mutational studies also revealed that different regions of DnaK that are critical for client recognition and binding (i.e., the α -helical lid and the binding groove) have a differential preference for the recognition of globally unfolded or near-native clients [91]. The finding that DnaK binds not only extended peptides but can be involved in stabilizing near-native conformations is significant, as it assigns previously undiscovered mechanisms of DnaK function that differs from the established paradigm.

Kellner *et al.* [133] used a combination of smFRET and microfluidics to look at the structural heterogeneity of non-native rhodanese in the presence of components of the DnaK/DnaJ/GrpE system. Binding of DnaJ alone to non-native rhodanese induced

significant heterogeneity between compact (high FRET) and expanded (low FRET) states of rhodanese. However, the addition of DnaK in the presence of ATP and sub-stoichiometric amounts of DnaJ caused a significant decrease in FRET efficiency that was indicative of a highly unfolded state. Kellner *et al.* also performed molecular dynamics simulations, based on experimental single-molecule data, to explore the stoichiometry of DnaK binding to rhodanese. It was suggested that rhodanese was saturated when 4–7 DnaK molecules were bound per rhodanese molecule, thus maximizing client expansion and minimizing the possibility of erroneous interdomain interactions. The forced unfolding of a client protein hence appears to be a generic mechanism of action of ATP-dependent chaperones [133–136].

Hsp60/GroEL

A landmark study on the GroEL system was one of the first attempts to monitor the folded-state of a client in real time upon interaction with a chaperone at single-molecule resolution [137]. This was achieved by observing the recovery of fluorescence of green fluorescent protein (GFP) as a reporter of the two kinetic steps of chaperonin-induced protein refolding. Similar methods, also employing GFP as a client, were employed by Takei *et al.* [28] to demonstrate that synchronous folding of clients can occur in both rings of GroEL/GroES, as opposed to the asynchronous mechanism previously suggested [138,139]. GroEL has been shown to populate two functional states, an asymmetrical GroEL:GroES₁ “bullet” complex and a symmetrical GroEL:GroES₂ “football” complex [26]. In independent experiments, the formation of these “bullet” and “football” complexes was synchronized via the release of caged-nucleotides (triggered by a UV flash) to measure the time taken for individual GFP molecules to refold. Not only did Takei *et al.* observe simultaneous folding of GFP in both rings of the “football” complex, but GFP also folded with the same kinetics as compared to folding in the conventional GroEL “bullet” complex [28,137]. During periods of cellular stress when the concentration of non-native species is high, the formation of the “football” complex is promoted [39]. Due to the enhanced ability of the “football” complex to fold multiple clients simultaneously (and thus a higher turnover of folded clients), its formation has been postulated to be a quality control mechanism that assists in maintaining proteome integrity. However, evidence for this hypothesis is lacking *in vivo*.

Sharma *et al.* [140] developed an smFRET system using a slow folding double mutant of MBP (DM-MBP) to enable the direct observation of GroEL-induced conformational changes of a protein at single-molecule resolution. Interestingly, they found that GroEL actually induces the unfolding of DM-MBP via a stretching

mechanism facilitated by the binding of the client across many spatially distinct apical domains of GroEL. Binding of ATP to GroEL then caused increasingly hydrophobic regions of DM-MBP to be sequentially released into the GroEL cavity, providing a mechanism by which GroEL assists protein folding by smoothing the folding landscape of the client. In a complementary study by Gupta *et al.* [136], a combination of photoinduced electron transfer and fluorescence correlation spectroscopy (PET-FCS) [141] was used to monitor the microsecond conformational dynamics of DM-MBP encapsulated in a GroEL nanocage. DM-MBP conformational chain dynamics were significantly restricted when confined in wild-type GroEL and its folding was accelerated. However, this was not the case when DM-MBP was confined in a cage formed by a mutated form of GroEL in which residues on the inside of the chamber were neutrally charged. From these experiments, it was concluded that GroEL (and by inference its human homologue, TriC/CCT) mediates active refolding of kinetically trapped proteins by (i) sterically confining entropically destabilized misfolded intermediates and (ii) establishing a charged hydrophilic environment to promote hydrophobic collapse. While these mechanisms had been previously suggested [134], it had not been experimentally demonstrated until these single-molecule techniques were applied [136].

Hsp90

Despite the significant amount of research into Hsp90 function, the precise molecular details regarding the role of ATP hydrolysis in regulating conformational changes of Hsp90 and the folding of its clients have remained largely elusive [55]. A seminal study by Mickler *et al.* [47] employed smFRET and TIRF microscopy to reveal significant conformational dynamics of Hsp90, even at saturating ATP concentrations. Mickler *et al.* was able to temporally observe rapid fluctuations in the FRET efficiency, corresponding to conformational changes between the “open” (low FRET) and “closed” (high FRET) state of Hsp90. The conformational dynamics observed occurred at time frames that were significantly faster than that of Hsp90-mediated ATP hydrolysis (typically ~100 s per ATP molecule) [47]. Thus, it appears that the observed conformational changes are only weakly coupled to ATP hydrolysis and are predominantly driven by stochastic (thermal) fluctuations. These findings have since been further corroborated in subsequent single-molecule studies [142,143], with increasingly sophisticated multi-color FRET experiments revealing for the first time directionality of the Hsp90 ATPase cycle [144].

In contrast to the above studies that addressed changes in the global conformation of Hsp90, Schulze *et al.* [145] focused more specifically on the role of

local conformational changes during the ATP hydrolysis cycle of Hsp90. Using single-molecule PET-FCS, Schulze *et al.* was able to monitor (at sub-nanometer resolution) the microsecond conformational dynamics of the NBD lid in response to ATP binding. Preliminary ensemble measurements suggested a two-state mechanism of lid closure, whereby extremely rapid reconfiguration of the lid over the nucleotide-binding cleft occurs followed by a slower maturation step to complete binding [145]. This finding was extended by the PET-FCS measurements, which indicated that the nucleotide lid was extremely mobile and fluctuated at sub-millisecond resolution in the apo (nucleotide-free) state of Hsp90 but was conformationally more restricted in the presence of ATP. The rapid fluctuations of the lid segment in the apo-Hsp90 state is thought to prime the lid over the nucleotide binding cleft so that it can rapidly close upon ATP binding. Of additional interest was the finding that the co-chaperone Aha1 greatly assists in the release of the nucleotide lid, adding an additional level of regulatory control to the functional cycle of Hsp90 [145]. While there has been significant attention on the conformational dynamics of Hsp90 and its regulation by ATP hydrolysis, there have been limited insights made into how these dynamics influence the conformation of its clients; this is an intriguing area for future studies.

Hsp100

To date, the majority of single-molecule chaperone studies have investigated the molecular function of the Hsp70/40, Hsp60 and Hsp90 classes of Hsp, while Hsp100 disaggregases have been studied less intensively. This was addressed recently by Okuda *et al.* [146], who for the first time attempted to observe the dynamic interactions between an aggregated client and the yeast Hsp104 machinery using single-molecule fluorescence microscopy. In this work, a variant of TIRF microscopy, termed highly inclined and laminated optical sheet (HILO) microscopy, was used to illuminate further into the sample volume (>150 nm) to observe larger luciferase aggregates. There was a higher percentage of co-localization observed between luciferase aggregates and an ATPase deficient Hsp104 mutant, which is unable to hydrolyze ATP (called Hsp104_{TRAP}), compared to the wild type Hsp104. Furthermore, Hsp104_{TRAP} was able to stably interact with aggregates for an extended period of time, suggesting the presence of high affinity interactions. In contrast, wild-type Hsp104 was observed to bind to the luciferase aggregates only transiently, with dwell times corresponding to a two-component reaction consisting of fast (~4 s) and slow (~30 s) steps that would normally be masked in ensemble measurements. The two kinetic steps were attributed to the dissociation of Hsp104 following hydrolysis of ATP within a single subunit (i.e., the fast

state) and the sequential hydrolysis of ATP within the hexameric Hsp104 ring (i.e., the slow state). Furthermore, when Hsp104 was in the presence of Ssa1 and Ydj1 (the yeast homologues of Hsp70 and Hsp40, respectively), a higher co-localization percentage was observed and the dwell times decayed at a lower rate (but were still characterized by the same two-component steps). The lower decay rate in the presence of Ssc1 and Ydj1 is rationalized by the partitioning of the reaction to the slow kinetic step (~ 30 s), which is thought to be the association in which Hsp104 is functionally active. Collectively, these results show that ATP-bound Hsp104 has a higher affinity for aggregated protein and that this affinity is enhanced in the presence of the Hsp70/40 system, likely due to the recruitment of clients to Hsp104 as has been suggested by ensemble-based measurements [118,147]. Furthermore, the results provide a mechanism by which co-chaperones facilitate disaggregase activity by encouraging multiple rounds of ATP hydrolysis for efficient translocation of clients through the Hsp104 pore. Subsequent experiments that employ the use of fluorescently labeled Ssc1 and

Ydj1, thereby facilitating direct observation of their interactions with the aggregates and Hsp104, would provide fascinating insights into the precise mechanisms by which these Hsps assist the disaggregase activity of Hsp104.

sHsps

The heterogeneous, dynamic and polydisperse nature of sHsps would appear to make them particularly amenable to study using single-molecule techniques. It is somewhat surprising then that they have received such little attention within the single-molecule field. While force spectroscopy techniques have been used previously to structurally characterize sHsps [148], it was not until recently that such techniques had been utilized to investigate sHsp function at the single-molecule level [117]. Ungelenk *et al.* [117] used optical tweezers to examine the effect of Hsp42 (a yeast sHsp) on the refolding, misfolding and aggregation of MBP and a four-subunit repeat of MBP (4MBP) (Fig. 6A). Mechanically unfolded 4MBP was returned to zero forces in the absence of Hsp42

Fig. 6. The effect of the sHsp Hsp42 on the refolding and aggregation of 4MBP as assessed by single-molecule force spectroscopy. (A) Schematic of the experimental setup. (B and C) First stretching-relaxation event with natively folded 4MBP. The WLC curves (gray lines) show the different states of 4MBP during unfolding (transitions from 4 → U represent the sequential unfolding of a single MBP domain). A colored dot denotes the presence of an observed structure and their abundance across many molecules is depicted in the pie chart. (D and E) Subsequent pulling of 4MBP in the absence of Hsp42. The presence of tight and weak aggregated 4MBP species that resisted unfolding can be observed. (F and G) Subsequent pulling of 4MBP in the presence of Hsp42. The presence of the aggregated species was significantly reduced and the formation of native-like structures was promoted. Figure adapted with permission from Ungelenk *et al.* [117].

(Fig. 6D and E), which resulted in the formation of aggregates that were unable to be unfolded again (termed tight aggregates, red dots) or unfolded in sections larger than that of a single MBP domain (termed weak aggregates, orange dots). In contrast, when stretched 4MBP was relaxed in the presence of Hsp42 the formation of tight aggregates was completely abolished and the occurrence of weak aggregates was reduced (Fig. 6F and G). Interestingly, secondary-pulling events resulted in the unfolding of native-like MBP domains, revealing that Hsp42 is able to minimize erroneous interdomain interactions and promote the formation of native structures. Similar experiments with a single MBP protein also indicated that Hsp42 does not interact with globally unfolded structures, but instead promotes and interacts with near-native structures to suppress their aggregation. These findings are congruent with those observed for the ATP-independent chaperone trigger factor, a non-Hsp chaperone that associates with the ribosome and interacts with nascent polypeptides [149]. The ability of sHsps to keep misfolding proteins in a native-like folding competent state is consistent with its proposed role in sequestering its clients into high-molecular-mass complexes to facilitate subsequent refolding by ATP-dependent chaperones [82,150].

Outlook

The significant heterogeneity and dynamic nature of Hsps has limited the study of their chaperone function using ensemble-based methods. However, in the past few decades, single-molecule techniques have emerged as an alternative method to study the precise molecular mechanisms by which Hsps fold and prevent the aggregation of proteins. Typically, single-molecule studies have primarily focused on the ATP-dependent chaperones, with novel observations emerging from these works that have helped shape the existing paradigms of their activity [133,136,144,151]. For instance, it is now known that Hsp70 can recognize different conformations of proteins [91] and that GroEL can unfold proteins prior to sequestration [140]. Despite the ever-growing amount of research into the mechanisms of ATP-dependent chaperones, some of the fundamental mechanisms by which they assist protein folding and prevent aggregation remain elusive. For instance, the effect of ATP hydrolysis on the conformation of bound clients and how this might assist protein folding are not well understood, particularly for Hsp90 and Hsp100. One key question within the field is whether chaperones promote protein folding by introducing structural and entropic constraints on the polypeptide that minimize non-native interactions or smoothen folding landscapes. Specifically, it is not known how chaperone binding affects the free energy of the polypeptide in order to guide it toward

a specific protein topology (e.g., proteins that contain a TIM-barrel fold often require folding assistance by Hsp60). Some computational and theoretical approaches have already been implemented to address this problem [152]; however, single-molecule techniques would be an ideal experimental approach to supplement these theoretical insights. While some single-molecule work has already been undertaken to interrogate the structural constraints and entropic effects induced by chaperones upon binding to their clients [133,136,140], further experiments are required to definitively describe the physical mechanisms that underpin chaperone function. Furthermore, the mechanism by which chaperones can transition between different functions is still unclear. For example, how does Hsp70 transition from assisting the folding of nascent polypeptides to its role as a component of the disaggregase machinery? Since Hsp70 is the central regulator of the Hsp network, it is essential to understand how Hsp70 interacts with various other Hsps to mediate different functional roles within the cell.

While there has been some work investigating the functional mechanisms of ATP-independent chaperones (e.g., trigger factor; reviewed in more detail in Refs. [153,154]), there has been comparatively little work done using single-molecule techniques to study sHsp function to date. We suggest that such approaches are ideally suited to investigate sHsp molecular chaperone action. For instance, the development of novel, fluorescence-based methods to study sHsp–client interactions, allowing the determination of on/off rates, stoichiometries and binding affinities. By combining fluorescence and force-based single-molecule techniques, which has been shown previously [155–157], fundamental aspects of sHsp chaperone function, typically inaccessible by ensemble measurements, can be interrogated.

Single-molecule techniques offer an exciting avenue by which the complex interactions between Hsps, co-chaperones and clients can be probed in exquisite detail. While to-date the majority of single-molecule studies have looked at the function of individual Hsps, a small number of studies have examined the effect of multiple components and co-chaperones on protein folding and chaperone function [133,144–146,158,159]. Notably, many studies that investigate the function of Hsp70 invariably examine the role of its co-chaperone Hsp40 in its chaperone function. However, the complexity of the chaperone systems studied using single-molecule techniques has thus far been limited, due to the difficulty in observing multiple chaperone/client components simultaneously. These studies typically examine the cooperativity of chaperone networks by employing “dark” components, which are elements of the chaperone network that are present but not able to be directly observed

via fluorescence or force spectroscopy. Regardless, these types of studies represent an important starting point for the study of Hsp networks that can, with further development, be applied to directly measure the dynamics that underpin the cooperative interactions between Hsp components.

A significant hurdle that faces all single-molecule fluorescence studies is that in order to observe the fluorescence signal from a single molecule, the background fluorescence originating from surrounding molecules in solution must be effectively eliminated. Consequently, the maximum effective concentration of labeled species is limited to a few tens of nM, which is often below the equilibrium dissociation constant (K_D) of most biochemical associations [22]. There is therefore a need to compromise between the low concentrations needed for visualization of single molecules and maintaining the high concentrations needed to accurately reconstitute physiologically relevant protein–protein interactions. This trade-off is particularly important in the context of Hsps as the concentrations of these chaperones are variable across cell types and drastically up-regulated during periods of cellular stress [160,161]. This “concentration problem” has traditionally been addressed by employing optical techniques such as TIRF microscopy [131], which reduces the excitable volume of the sample in order to significantly reduce background fluorescence and improve the signal-to-noise ratio. More recently, technologies such as micro/nanofluidic devices or nanovesicles, which can also be used in conjunction with single-molecule optical microscopes, reduce the reactant volume and allow for the observation of single molecules at higher effective protein concentrations [162,163]. For example, Miyake *et al.* [164] implemented a microfluidic nanohole array that allowed for the single-molecule observation of GroEL/GroES binding kinetics at high concentrations (~500 nM) of labeled GroES. Furthermore, novel fluorescent-labeling approaches such as PhADE (PhotoActivation, Diffusion, and Excitation) allow for higher concentrations of labeled protein to be present in the reaction volume during imaging [165]. This background reduction is achieved by having a protein of interest in solution that is fused to a photo-activatable fluorescent protein that is only activated using a second excitation wavelength and fluorescence imaging performed after a time delay. Activated molecules that are not bound to their surface-immobilized target diffuse out of the observation volume before the imaging takes place, thereby decreasing the background fluorescence and allowing for only the detection and characterization of molecules that are bound to the target protein. The ability of PhADE to investigate dynamic protein systems has been demonstrated in the DNA-replication field, where single replication proteins have been visualized on DNA templates at concentrations up to 2 μ M [165]. The continued implementation and development of these techniques will help to overcome the “concentration

problem” and enable the study of chaperone function at higher concentrations than has been achieved in single-molecule experiments to date. As single-molecule techniques continue to develop as a powerful tool to study dynamic protein systems, using them to study Hsps in conjunction with other biophysical, chemical and structural methods will enable a greater understanding of this dynamic chaperone network.

To date, all single-molecule studies examining Hsp function have done so using reconstituted protein systems *in vitro*. Consequently, these experiments do not accurately reproduce the crowded environmental conditions in which Hsps function within the cell. While such *in vitro* studies provide critical insights into the mechanisms by which Hsps function as chaperones, it is also important to take the knowledge acquired from these single-molecule studies and apply them for the study of Hsps within the cellular environment. The ability to transfer the study of complex biological machineries from single-molecule *in vitro* systems to within living organisms has been demonstrated for many processes, with DNA replication and repair as a notable example [166]. Here, *in vitro* studies that determined the molecular details of DNA replication (e.g., the dynamic exchange and processivity of DNA polymerases) [167] have been supplemented by single-molecule *in vivo* imaging of the spatio-temporal organization of these machineries within living cells [168]. This development is highlighted by the novel finding that error-prone polymerases are spatially regulated in *E. coli* via sequestration to cellular membranes until they are required by the cell [169], a regulation mechanism that could not have been identified using reconstituted components *in vitro*. Critically, the complementary nature of *in vitro* and *in vivo* experiments has provided a greater understanding of how DNA replication and repair occurs and can be regulated. Therefore, by applying the knowledge acquired from *in vitro* studies, we will be able to begin to interrogate the Hsp network *in vivo* using cell-based single-molecule techniques and thereby elucidate how these critical biological machines are organized and maintain proteostasis. Finally, by employing a combination of single-molecule and other biophysical methods to study Hsp networks, we may begin to understand how these networks function cooperatively and why they fail to prevent protein aggregation in the context of diseases.

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Abbreviations used:

4MBP, four-subunit repeat of MBP; ACD, α -crystallin domain; DM-MBP, double mutant of MBP; *E. coli*, *Escherichia coli*; GFP, green fluorescent protein; HILO microscopy, highly inclined and laminated optical sheet microscopy; Hsps, heat-shock proteins; MBP, maltose binding protein; NBD, nucleotide-binding domain; NEF, nucleotide-exchange factor; PET-FCS, photoinduced electron transfer and fluorescence correlation spectroscopy; PhADE, PhotoActivation, Diffusion, and Excitation; SBD, substrate-binding domain; sHsps, small heat-shock proteins; smFRET, single-molecule fluorescence resonance energy transfer; TIRF, total internal reflection fluorescence; TPR, tetratricopeptide repeat.

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