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Small Heat Shock Proteins (sHSPs) are one of the least well understood classes of molecular chaperones, proteins which act to prevent or reverse improper protein associations (1). The importance of the sHSPs is evidenced by their almost ubiquitous expression (2), the presence of multiple sHSP genes in most organisms (3), and their dramatic up-regulation under stress conditions making them among the most abundant of cellular proteins (4). They are implicated in a range of disease states including cataract, cancer, myopathies, motor neuropathies, and neurodegeneration (5–8). The current view of their chaperone action is that they bind unfolding “client” proteins, thereby preventing their irreversible aggregation (9–12). These sHSP:client complexes then interact with ATP-dependent chaperones to allow refolding of the clients (9–12). Structural interrogation of the complexes they form with clients has however been hampered by their apparent heterogeneity, and their organization remains consequently very poorly defined (13–15).

MS is an emergent technology for the structural biology of biomolecular systems, including those complicated by polydispersity (16), allowing the interrogation of a wide range of clients. It therefore appears that the sHSPs act as an extensive and plastic chaperone ensemble, facilitating their protection of a wide range of clients.

Results

A Transition from Monodisperse Dodecamer to Polydisperse Ensemble Occurs at Heat Shock Temperatures. To investigate thermally regulated changes in quaternary organization we obtained nanoelectrospray (nES) mass spectra of HSP18.1 at a range of temperatures (Fig. 1A). At 22 °C we observe a single discrete charge state series centered around a 34+ ion at 6,350 m/z, corresponding to a dodecamer of 216,017 Da. This is in accord with our previous analytical ultracentrifugation (20) and MS (23) measurements, as well as the crystal structure of the closely related HSP16.9 from wheat (24). As the temperature of the solution is increased we observe a reduction in signal corresponding to dodecamer, and new signal appearing both below 3,000 m/z and above 7,000 m/z. The former corresponds to populations of monomer and dimer, and the latter to a range of oligomeric sizes, from 13 to 20-mer (Fig. S1). Therefore at elevated temperatures HSP18.1 dodecamers appear to both dissociate into suboligomeric species, and be augmented to form higher-order oligomers.

To interrogate these thermally regulated changes in detail we determined the relative populations of these species and, thereby, the allocation of the constituent subunits amongst the different oligomeric states (Fig. 1B). This demonstrates a clear change in protein partitioning at heat-shock temperatures, with most of the

HSP18.1 sequence is responsible for binding clients (22), but the quaternary organization of the resulting sHSP:client complexes has remained elusive.

Here we employ a combination of thermo-controlled, time-resolved, and tandem-MS approaches to elucidate molecular details of complex formation. We find that the dodecameric HSP18.1 undergoes both dissociation into suboligomeric species, and expansion into high-order oligomers at heat-shock temperatures. Upon the addition of Luc, large and polydisperse sHSP:client complexes are rapidly formed. We monitor the kinetics of this reaction, and show it to be bimodal, revealing distinct “binding” and “augmentation” phases. Our tandem-MS approach allows us to identify and quantify the individual complexes which comprise the heterogeneous sHSP:client ensembles, uncovering that they are comprised of a variable number of both HSP18.1 and Luc. We observe over 300 different complex stoichiometries, revealing a remarkable diversity of interaction between sHSPs and their clients. It therefore appears that the sHSPs act as an extensive and plastic chaperone ensemble, facilitating their protection of a wide range of clients.
protein being reallocated from dodecamers into higher-order oligomers. Quantification of the number of monomers populating the different higher-order oligomeric states shows that those composed of an even number of subunits are clearly favored over those with an odd number (Fig. 1C). The resulting “saw-tooth” distribution, along with the increasing presence of free monomers and dimers at heat-shock temperatures, suggests that higher-order oligomers result from the dissociation of dodecamers into suboligomeric species, which subsequently recombine with oligomeric species. Such a mechanism for oligomeric rearrangement is consistent with the rate-limiting step in HSP18.1 subunit exchange being dissociation of the dodecamer (23), and, through the increased amount of suboligomeric species at elevated temperatures, provides a rationale for the temperature-dependence of subunit exchange (13, 25). These thermal rearrangements in quaternary organization are reversible (Fig. S2). Interestingly, the polydisperse and saw-toothed distribution we observe is very similar to the distribution of oligomers populated by the mammalian sHSPs αA- and αB-crystallin at ambient conditions (26), raising the possibility that a polydisperse ensemble of oligomers may be a widespread feature of functional sHSPs.

Thermally Induced Changes Are Underpinned by Quaternary Protein Dynamics. To extract thermodynamic parameters for these thermal rearrangements we considered the equilibrium of a single protein subunit between its free state in solution, and its bound state within an oligomer. As such we can define an equilibrium constant, and hence an average subunit free energy (ΔGAV, Supporting Information Methods). The free energies are found to scale linearly with temperature, allowing elucidation of the enthalpic and entropic components to the free energy (Fig. 1D, Fig. S3). For the dodecamer these values were determined as ΔHAV = −184 ± 12 kJmol⁻¹ and ΔSAV = −570 ± 40 Jmol⁻¹ K⁻¹, and for the dimer ΔHÅV = −22 ± 2 kJmol⁻¹ and ΔSAV = −84 ± 7 Jmol⁻¹ K⁻¹. The linear scaling of free energy with temperature shows that the temperature dependence of the oligomer distributions can be explained simply by the interplay of the stabilities of a single set of structures, and that it is unnecessary to propose that the individual oligomers themselves undergo large structural rearrangements at heat-shock temperatures. This surprising result is contrary to what has been observed for HSP26 from yeast, in which a conformational change of its “middle domain” underlies the thermal activation of its chaperone function (27). The presence of such a middle domain however appears unique to HSP26 (27), and is certainly absent in HSP18.1 (12). Our results here indicate that, at least for HSP18.1, activation is mediated not by an alteration in secondary or tertiary structure, but rather a dynamical change in quaternary organization that results from the varying thermodynamic stabilities of the individual sHSP oligomers.

Protection of Client Occurs Through the Bimodal Formation of an Ensemble of Polydisperse Complexes. To examine its chaperone function, we obtained MS spectra of HSP18.1 incubated at 42 °C for varying times with the model client Luc, at a 1:1 molar ratio of HSP18.1 (dodecamer) and Luc (monomer) (Fig. 2A). Before heating, major charge state envelopes are observed around 4,000 and 6,500 m/z, corresponding to unbound Luc monomer and HSP18.1 dodecamer respectively. After as little as 30 s of incubation the concentration of free Luc decreases, and concurrently a broad and largely unresolved area of signal is observed above 8,000 m/z, indicating the formation of sHSP:client complexes. This signal increases with longer incubation and also shifts to higher m/z, showing that not only are more complexes formed but also that they become more massive.

By quantifying the relative abundances of these different species, we can extract their rates of disappearance or formation (Fig. 2B). We find that the decay of Luc is fitted by a single exponential, giving a rate constant of 2.41 ± 0.32 min⁻¹. The formation of complex however is fitted by a biexponential, with an initial fast phase (2.27 ± 0.13 min⁻¹) and a subsequent slow phase (0.11 ± 0.03 min⁻¹), which is mirrored by the decay of HSP18.1 dodecamer. From the mean m/z of signal corresponding to complex we can estimate a mean mass (Fig. S4), and we find that this also follows biexponential kinetics, with rate constants of 1.83 min⁻¹ and 0.05 min⁻¹. Together, this demonstrates that sHSP:client complex formation has two distinct stages, an initial binding phase, in which client is bound and aggregation prevented, and a subsequent augmentation phase. This second stage involves the incorporation of additional sHSP, presumably to reach optimal stability of the complex.

Tandem Mass Spectrometry Allows the Deconvolution of the Polydisperse Ensemble of Chaperone:Client Complexes. The signal at high m/z is characteristic of a polydisperse ensemble, the many
components of which give rise to extensive peak overlap, frustrating attempts at spectral interpretation. We have previously developed a tandem-MS methodology to allow the interrogation of heterogeneous systems (28). We first examined a 1 : 0.1 mixture of HSP18.1 and Luc which had been incubated at 42 °C for 10 min, with the resulting complexes separated from the remaining HSP18.1 by size exclusion chromatography (SEC) (Fig. S5). We chose this ratio to ensure complete protection of Luc while keeping the entire range of complexes amenable for MS interrogation. An MS spectrum of the resulting complexes displays a broad area of signal from 8,000 to 13,000 m/z (Fig. 3A). Ions comprising the most intense complex peak, at 8,950 m/z, were selected and activated by energetic collisions with argon atoms, resulting in the appearance of signal at both low and high m/z (Fig. 3B). The former corresponds to HSP18.1 monomers, and the latter to species having been stripped of one (10,000–16,800 m/z) or two monomers (19,000–30,000 m/z) (Fig. S6). This asymmetric dissociation of the oligomers is a general feature for activated protein complexes in the gas phase (29), and leads to an effective charge reduction of the species being interrogated (30). The resulting increased separation between charge states is such that masses can be measured that are accurate enough to determine unambiguously the stoichiometries of the sHSP:client complexes (Fig. S7). Assignment and relative quantification of the different species reveals, for this isolation, a polydisperse ensemble of HSP18.1 binding one Luc client (Fig. 3C). Analogous tandem-MS experiments were performed on the different peaks in the MS spectrum corresponding to HSP18.1:Luc complex (Fig. S8), and results combined to provide a comprehensive view of the oligomers comprising the polydisperse ensemble. At this ratio we identified complexes containing one Luc and as few as 14, or as many as 25, HSP18.1 subunits. Furthermore, we find that complexes with an even number of sHSP subunits bound to a client are more abundant than those with an odd number. This mirrors the saw-tooth pattern observed for HSP18.1 at elevated temperatures in the absence of client (Fig. 1C). This strongly suggests that the dissociation of dodecamers and formation of higher-order oligomers, via thermally regulated quaternary dynamics, constitutes a molecular activation integral to the chaperone function of HSP18.1.

Dimers and Monomers Mediate Different Stages of Complex Formation. To examine the initial stages of complex formation we performed similar tandem-MS experiments after only 30 s of incubation, at a 1 : 1 ratio (Fig. 4A). We selected regions at low m/z, corresponding to small, and hence early, complexes for dissociation. In this series of experiments we observe a number of different higher-order sHSP oligomer and sHSP:client complex stoichiometries. The presence of both these two classes of species, as well as unbound Luc and HSP18.1 dodecamer, confirms that these conditions allow us to examine the early phase of complex formation. Notably, despite tandem-MS interrogation of the entire region in the mass spectrum where a hypothetical [HSP18.1]₂[Luc] complex would appear (7,000–8,500 m/z, ...
reveals the distribution of all HSP18.1:Luc complexes formed at a 1:1 ratio. An expansion of the [HSP18.1]_x,[Luc]_x region is shown. A range of complexes and higher-order oligomers are identified, with the labelling referring to the parent oligomer. Theoretical peak positions arising from dissociation of [HSP18.1]_x,[Luc]_x are indicated by dashed white lines, and do not correspond to the charge state series observed. This suggests this species is not formed during protection of Luc. A Quantifying the stripped oligomers shows that the abundance of complexes with an even number of subunits is higher than those with an odd number at all time points. However this difference is greatest at earlier time points. Overlaying the parameters determined in Fig. 2B for the binding and augmentation steps in complex formation gives an excellent fit, suggesting that dimers are involved in the client binding process.

Fig. S4), we find no evidence for this species. Though qualified by our limit of detection (5% of the most abundant peak in a single tandem-MS spectrum), this suggests that the HSP18.1 dodecamer is not directly involved in substrate binding. Instead, it seems the role of the dodecamer is as a reservoir of HSP18.1 subunits which can then set up the polydisperse ensemble for substrate binding.

We performed additional tandem-MS experiments at 8,250 m/z, as in the top panel of Fig. 4A, but at different incubation times. From quantifying the abundance of the resulting complexes it is apparent that at the complexes with an even number of HSP18.1 subunits are more abundant than those with an odd number. Moreover, this disparity is largest at the earliest time points (Fig. 4B). Plotting the relative abundance of these “even” complexes versus the “odd” ones, and applying the bieponential parameters resulting from the binding and augmentation phases (from Fig. 2B), gives an excellent fit. Taking this together with our previous observation that the HSP18.1 dodecamer undergoes a rapid exchange of subunits, with dimers exchanging faster than monomers (23), strongly suggests that dimers play a crucial role in initial complex formation, and that the slower rearrangements to achieve optimal stability are additionally mediated by the movement of monomers.

The Polydisperse Ensemble of Complexes Contains Variable Numbers of Both Chaperone and Client. To examine the full distribution of complexes formed at this 1:1 sHSP:client ratio we performed similar experiments on a fraction (11.5–12.3 mL) isolated by SEC (Fig. 5A). Remarkably we are able to identify unambiguously and quantify species containing 14 to 36 HSP18.1 subunits, and 1 to 3 Luc (Fig. 5B). Furthermore, analogous experiments performed with another client protein, citrate synthase, reveal a similar range of binding stoichiometries (Fig. S9). Therefore we can deduce that sHSP:client complexes can contain a variable number of both chaperone and client components.

By convoluting these tandem-MS-derived distributions for this SEC fraction with the whole SEC peak (Fig. S10) we extrapolated the complete distribution of complexes formed at this particular sHSP:client ratio (Fig. 5C). The range of complexes formed is striking in its heterogeneity. We observe over 300 different HSP18.1:Luc combinations being populated to greater than 1% of the population of [HSP18.1]_x,[Luc]_x, the most abundant complex under these conditions (Fig. 5C). This reveals why characterization of the complexes formed between sHSPs and clients has proven elusive by using traditional structural biology approaches, and highlights the utility of MS for studying polydisperse protein ensembles. Moreover, while ATP-dependent molecular chaperones have strict ratios of interaction with their clients (31–33), the picture that emerges here for the ATP-inde-
pendent sHSPs is the complete inverse: they appear defined by the diversity of their interaction.

**Discussion**

The Chaperone Action of HSP18.1 is Regulated by Quaternary Protein Dynamics. Here we have found that HSP18.1 exists as a dodecameric oligomer in equilibrium with dimers, monomers, and higher-order oligomers (Fig. 6A). At elevated temperatures, where HSP18.1 performs its protective role, these equilibria shift such that the majority of the protein is partitioned to these higher-order oligomeric states. Upon the addition of unfolding client, complexes are formed, holding the client stable relative to precipitation (Fig. 6A). Notably multiple higher-order oligomer states are observed, and the complexes are remarkable in their polydispersity, varying in both the number of both sHSP and client subunits. This is surprising considering HSP18.1 exists as a monodisperse dodecamer at ambient conditions. Moreover, considering that no complexes are observed containing 12 or fewer HSP18.1 subunits, this strongly indicates that the dodecamer is not directly involved in the binding of client protein. Rather it appears that the dodecamer exists as a reservoir for suboligomeric species which are released under heat-shock conditions, and can assemble with residual oligomers to form higher-order oligomers. This thermally activated dynamical change in oligomerisation thereby produces the active chaperoning ensemble of the sHSP. Stress-regulated switching between multiple functions, with higher-order oligomers displaying ATP-independent chaperone activity, has been observed for enzymes in yeast (34) and Arabidopsis (35). It may be that the dodecameric form of HSP18.1 is chaperone-inactive simply to prevent unproductive associations with protein chains under recovery conditions. In light of the multiple functions of the α-crystallins (36, 37), vertebrate members of the sHSP family, the fascinating possibility that HSP18.1 performs a cellular role additional to its chaperone function should not be discounted.

We have shown here that it is these quaternary dynamics which are the molecular basis of HSP18.1 chaperone function. Moreover, rather than relying on ATP, posttranslational modification, or large structural changes, these dynamics are temperature controlled. The importance of intrinsic dynamic fluctuations in secondary and tertiary structure for protein function has become established in recent years (38–40), a paradigm that is extended to the quaternary level by the dynamic regulation of chaperone function described here.

Polyp dispersity and Subunit Exchange Establish an Extensive and Plastic sHSP Chaperone Ensemble. We have identified over 300 different stoichiometries of interaction between HSP18.1 and Luc, all stemming from a single “parent” form, the dodecamer. This switch from monodisperse to polypolysome at heat-shock temperatures effectively establishes an ensemble of chaperones. In addition, another consequence of the dynamic quaternary structure of sHSPs is the formation of heterooligomers by coassembly of monomers immediately after their biosynthesis, forming an equilibrium of species maintained via subunit exchange (23–25). In pea another dodecameric cytosolic sHSP, HSP17.9, has been identified which is of the same evolutionary class (19), and hence capable of forming hetero-oligomers with HSP18.1. The pea genome remains incompletely sequenced, but it is likely that more sHSPs compatible with HSP18.1 remain to be discovered, as the number of equivalent sHSPs ($N_{\text{equ}}$) in Arabidopsis ($N_{\text{HSP}}$ = 6), rice ($N_{\text{HSP}}$ = 8), and Californian poplar ($N_{\text{HSP}}$ = 18) are considerably greater (41). Using these numbers, we can determine the maximum number of possible combinations of hetero-oligomers ($N_{\text{comb}}$) for the different organizational states of HSP18.1 we have observed here (Fig. 6B).

$N_{\text{comb}}$ scales exponentially with $N_{\text{HSP}}$ such that, based on the complexes seen here for HSP18.1, if $N_{\text{HSP}} = 18$ over $10^{12}$ combinations are possible! Though factors such as tissue-specific expression levels need to be considered, this provides a glimpse of a remarkably disperse sHSP chaperone ensemble. This is reminiscent of the immune system in higher eukaryotes, whereby a relatively small number of genes (approximately 300) can give rise to over $10^{6}$ different antibodies, allowing the recognition of the diverse structures of antigens (42). Plants are particularly prone to quotidian cycles of heat stress, and the resulting protein aggregation represents a major insult to maintenance of cellular protein homeostasis, or proteostasis (43). It is tempting to speculate that evolution of such an extensive sHSP ensemble, within the context of the wider chaperone network, allows plants to protect themselves against the diversity of unfolding client proteins, thereby maintaining proteostasis.

**Materials and Methods**

Mass Spectrometry. Mass spectra were obtained on an liquid chromatography time-of-flight (LCT) or quadrupole time-of-flight (Q-ToF) 2 (both Waters) according to a previously described protocol (44). A custom-built nES probe was employed for the spectra of HSP18.1 at different temperatures as described previously (45). In all cases the buffer was 200 mM ammonium acetate, pH 6.8.

The following instrument parameters were used on the LCT: nES capillary 1.6 kV, sample cone 160 V, extractor cone 40 V, ion transfer stage pressure 9.3 mbar and 2.1 × 10⁻⁶ mbar ToF analyser pressure. The following parameters were used on the Q-ToF 2: nES capillary 1.6 kV, sample cone 160 V, extractor cone 40 V, accelerating voltage into the collision cell 30 V, ion transfer stage pressure 9.6 × 10⁻⁶ mbar, quadrupole analyser pressure.

Quantiitave and Thermodynamic Analysis of HSP18.1. The relative abundances of the different oligomers in a particular spectrum were obtained from the respective peak heights, an effective means for assessing the populations of species comprising polydisperse ensembles (28), and corrected for the m/z dependence of detector efficiency (47). From these abundances we calculated a stability constant and free energy difference between a subunit in its free and bound state (see Supporting Information Methods).

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