Quaternary dynamics and plasticity underlie small heat shock protein chaperone function

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Small Heat Shock Proteins (sHSPs) are a diverse family of molecular chaperones that prevent protein aggregation by binding clients destabilized during cellular stress. Here we probe the architecture and dynamics of complexes formed between an oligomeric sHSP and client by employing unique mass spectrometry strategies. We observe over 300 different stoichiometries of interaction, demonstrating that an ensemble of structures underlies the protection these chaperones confer to unfolding clients. This astonishing heterogeneity not only makes the system quite distinct in behavior to ATP-dependent chaperones, but also renders it intractable by conventional structural biology approaches. We find that thermally regulated quaternary dynamics of the sHSP establish and maintain the plasticity of the system. This extends the paradigm that intrinsic dynamics are crucial to protein function to include equilibrium fluctuations in quaternary structure, and suggests they are integral to the sHSP’s role in the cellular protein homeostasis network.

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. L4). At 22°C we observe a single discrete charge state series centered around a 34+ ion at 6350 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 HSP: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 indentify and quantify the individual complexes which comprise the heterogenous HSP: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.


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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 (ΔG°, 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 ΔH°AV = −184 ± 12 kJ mol⁻¹ and ΔS°AV = −570 ± 40 J mol⁻¹ K⁻¹, and for the dimer ΔH°AV = −22 ± 2 kJ mol⁻¹ and ΔS°AV = −84 ± 7 J mol⁻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 re-arrangements 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). After 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.

**Fig. 2.** Time-course of the formation of complexes between HSP18.1 and unfolding Luc. A 10-min incubation with HSP18.1 (6, 350 m/z) at 42 °C results in a decrease in the abundance of Luc (≈4, 100 m/z), and the concomitant appearance of signal at high m/z (≈8, 000 m/z) corresponding to sHSP:client complexes. Data are normalised such that the HSP18.1 is displayed at an intensity of 100%. Coloured boxes refer to isolations for tandem-MS experiments in Figure 4. B Quantifying the relative abundances of the different species shows the initial rapid binding of Luc by HSP18.1, followed by further incorporation of HSP18.1 into the resultant complexes. The average mass of these complexes (Red, right-hand 3-dimensional) mirrors this behavior, revealing the presence of distinct “binding” and “augmentation” steps in the chaperone action of HSP18.1.

**Fig. 3.** Identification and quantification of HSP18.1:Luc complexes by means of tandem-MS. A Spectrum of a complex formed between HSP18.1 and Luc at a 1:0.1 ratio. Unbound dodecamer is observed around 6, 350 m/z, and a broad area of signal, characteristic of a polydisperse ensemble, is observed above 8,000 m/z. B Selection of ions at a particular m/z, gray, and activation of selected ions leads to the removal of highly charged HSP18.1 subunits (≈1, 800 m/z) from the parent oligomers. Complementary stripped complexes appear at high m/z, sufficiently separated that they can be assigned unambiguously (Fig. S8). C Relative quantification of the different complexes from the heights of the peaks reveals a number of different stoichiometries of HSP18.1 bound to one Luc, and a dominance (p < 0.01) of those containing an even number of HSP18.1 subunits relative to a Gaussian distribution. This saw-tooth pattern mirrors that observed when HSP18.1 is heated on its own, (Fig. 1C), revealing the importance of temperature controlled dynamics of the sHSP in complex formation. Data and error bars represent the mean and standard deviation of three experiments.
reveals the distribution of all HSP18.1:Luc complexes formed at a 1:1 sHSP:client ratio. An expansion of the [HSP18.1]_{12}/[Luc]_1 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]_{12}/[Luc]_1 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.

From quantifying the abundance of the resulting complexes (Fig. 2C), 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 biexponential 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]_{12}/[Luc]_1, 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-
ependent 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.

Polydispersity 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 polydisperse 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_{sHSP}) in Arabidopsis (N_{sHSP} = 6), rice (N_{sHSP} = 8), and Californian poplar (N_{sHSP} = 18) are considerably greater (41). Using these numbers, we can determine the maximum number of possible combinations of hetero-oligomers (N_{comb}) for the different organizational states of HSP18.1 we have observed here (Fig. 6B).

N_{comb} scales exponentially with N_{sHSP} such that, based on the complexes seen here for HSP18.1, if N_{sHSP} = 18 over 10^{17} 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^{8} different antibodies, allowing the recognition of the diverse structures of antigens (42). Plants are particularly prone to quotidians 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: 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^{-3} mbar, quadrupole analyser pressure.

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**Fig. 6.** sHSPs establish a disperse chaperone network. A. The dodecameric form of HSP18.1 represents a “storage” form, which is in equilibrium with suboligomeric species, and, through recombination with these, higher-order oligomers. These higher-order species are themselves continually recycling through the loss and reincorporation of dimers and monomers. Upon heat-shock the equilibria shift (Red) from the dodecamer to dissociated species, and unfolding clients are bound to form sHSP:client complexes. B. From the number of different species we observe here for the different states of HSP18.1 (Orange) we can extrapolate to systems incorporating multiple sHSPs (Bars). Based on the ability of related sHSPs to hetero-oligomerise, the number of potential species (N_{comb}) can be calculated using the inset equation, where N_{sHSP} is the number of compatible sHSPs, i the number of HSP18.1 subunits in the oligomer, and N_{comb} the number of different bound states for a particular i. This reveals a remarkably extensive potential sHSP network, presumably catering for the array of different unfolding clients requiring protection from aggregation during cellular stress.
1.0 × 10⁻⁵ mbar, and ToF analyser pressure 8.1 × 10⁻⁶ mbar, with the collision cell pressurised to 35 μbar (argon). For tandem-MS the accelerating voltage was raised up to 200 V.

**Proteins.** HSP18.1 from *P. sativum* was expressed in *E. coli* and purified as described previously (20). Recombinant luciferase from *Photorhabdus luminescens* (New England Biolabs, Catalog E1701) Samples were buffer-exchanged using SEC at 6 °C (Superdex 200 HR10/30, GE Healthcare) or centrifugal concentrators (Vivaspin, Sartorius), and adjusted to the desired concentration using theoretical (46) extinction coefficients of 198,000 (HSP18.1 dodecamer) and 39,810 (Luc monomer).

To form complexes, HSP18.1 was incubated with Luc at 42 °C in a water bath for the desired time. The reaction was quenched on ice, which arrests complex formation but does not cause complex disassembly (13). Complexes were either formed in 200 mM ammonium acetate (pH 6.8), or buffer A (150 mM KCl, 5 mM MgCl₂, 2 mM Hepes, 2 mM DTT, pH 7.5) as stated in the text. For the experiments assessing the kinetics of complex formation (Fig. 2), the concentrations of HSP18.1 (dodecamer) and Luc (monomer) were using theoretical (46) extinction coefficients of 198,000 (HSP18.1 dodecamer)

**SEC at 6 °C (Superdex 200 HR10/30, GE Healthcare)** or centrifugal concentration (Vivaspin, Sartorius), and adjusted to the desired concentration using theoretical (46) extinction coefficients of 198,000 (HSP18.1 dodecamer) and 39,810 (Luc monomer).

The relative abundance 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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**Quantitative 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).
Supporting Information

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SI Text

Methods. Thermodynamic analysis of HSP18.1 oligomerization. There are two reasonable methods for considering the relative stability of the oligomers, where the concentration of the ith oligomer is given by \([P_i]\), and the concentration of monomers forming isomers is given by \([iP]\). The ‘step-wise’ free energy method \((\Delta G_{ST})\) is based on sequential equilibria between protein oligomers of the form \(P_i + P_{i-1} \rightleftharpoons P_i\), with the corresponding free energy for each oligomer given by \(\Delta G_{ST} = -RT \ln \frac{[P_i]}{[iP]}\).

Alternatively, we can consider the relationship between a protein oligomer with its constituent monomers according to the equilibria \(iP_i \rightleftharpoons P_i\), to allow us to directly compare the relative stabilities of all oligomers, with respect to the concentration of free monomers. The corresponding ‘average free energy per monomer’ is given by \(\Delta G_{AV} = -RT \ln \frac{[P_i]}{[i]}\), which reveals the difference between a monomer in solution and its bound state. This quantity is entirely independent of the formation mechanism, relying solely on the equilibrium concentration of the oligomer of interest, and the concentration of free monomer.

The concentration of each oligomer can be expressed as a function of the equilibrium constants and the concentration of free monomer according to either \([P_i] = P_iK_{i,0}\) or \([P_i] = \Sigma_{i=1}^{n_{oligo}}K_{ST,i}[P_i]^n\), where \(K_{0,1} = K_{ST,1} = 1\). The two equilibrium constants are therefore related through: \(K_{ST,i} = \Sigma_{i=1}^{n_{oligo}}K_{ST,i}[P_i]^{n_{oligo}}\).

In such a system it is important to distinguish between the total oligomer concentration, \(\Sigma_{i=1}^{n_{oligo}}[P_i]\), and the total concentration of monomers in the system, \(\Sigma_{i=1}^{n_{oligo}}[iP]\). While the former varies with temperature-induced changes in the equilibrium size distribution, the latter does not and can be defined as \(\Sigma_{i=1}^{n_{oligo}}[iP] = \Sigma_{i=1}^{n_{oligo}}iK_{AV,i}[P_i] = \Sigma_{i=1}^{n_{oligo}}\Sigma_{i=1}^{n_{oligo}}K_{ST,k}[P_i]^k\). From a complete set of equilibrium constants \([P_i]\) and hence the concentration of subunits partitioned into this oligomeric state \([iP]\) can be determined.

By plotting \(\Delta G\) versus \(T\) we obtained \(\Delta H\) and \(\Delta S\) values for both the stepwise and average quantities. In the case of data presented here, the reduced \(\chi^2\) values \(\chi^2/(N-p)\), where \(N\) is the number of data points, and \(p\) is the number of parameters, and \(\chi^2 = \Sigma N \left( \frac{\Delta G_{calc} - \Delta G_{exp}}{\Delta G_{exp}} \right)^2\) were determined to be in the range 1–1.5 when fitting to the linear model of \(\Delta G = \Delta H - T\Delta S\). We compared this to a more complex model \(\Delta G = \Delta H^0 + \Delta C_P (T - T_0) - T(\Delta S^0 + \Delta C_S \ln(T/T_0))\), where \(T_0\) is a reference temperature, \(\Delta H^0\) and \(\Delta S^0\) are the enthalpy and entropy changes at this temperature, and \(\Delta C_P\) is the change in heat capacity. An F-test between the fits to these models gave \(p\) values between 0.06 and 0.25 indicating that our measurements do not detect significant variation in heat capacity over the temperature range studied.

Identification of higher-order oligomers of HSP18.1. At elevated temperatures HSP18.1 forms a range of species at higher m/z than the native dodecamers. To identify the various oligomers we used a combination of the observed m/z values (A) and tandem-MS experiments. In these tandem-MS experiments we isolated the peak of interest in the quadrupole analyzer of the Q-ToF mass spectrometer, and removed highly charged monomers from the oligomers by collision induced dissociation (1). For example, after isolation and dissociation of the species comprising the peak at 7,380 m/z (B), we observed monomer at low m/z, and signal at high m/z corresponding primarily to a species of 269,775 Da, consistent with [HSP18.1]15. As this species must arise from one having been stripped of a single monomer, the original oligomer corresponds to a [HSP18.1]16. A minor amount of [HSP18.1]13, hence from [HSP18.1]14, is also observed. This process was then repeated for different peaks in the MS spectrum, thereby allowing us to identify all the oligomers which comprise the polydisperse ensemble of HSP18.1 at elevated temperature. Relative abundances were calculated from the peak heights in the MS spectrum, taking into account the contributions from the individual oligomers to the different peaks in the spectrum.
Reversibility of changes in HSP18.1 oligomerization. To assess the reversibility of the thermally regulated changes in oligomerization of HSP18.1 we raised the temperature of the solution from 22 °C to 45 °C, and cooled it back down to 28 °C, while monitoring it continuously in real time using a customized nanoelectrospray probe (2). As the temperature increases the relative amount of dodecamer decreases, and concomitantly higher-order oligomers are formed. As the temperature of the 45 °C solution is reduced this shift is reversed, such that at 28 °C the dodecamer again dominates the spectrum. This cooling process took approximately 15 min. This demonstrates that the thermo-dynamically controlled changes in oligomerization are rapidly reversible. Data are normalized such that the dodecamer remains at 100%.
Fig. 53. (A) Thermodynamic analyses of the oligomers. Stepwise (A), and average (B), free energies were determined and analyzed according to $\Delta G = \Delta H - T \Delta S$ for the different oligomers populated by HSP18.1 at different temperatures (Fig. 1). In the case of the 12mer, as the concentration of oligomers of size between 3 and 11 was too low to be detected, the calculated $\Delta G_{ST}$ is defined through the equilibrium constant $K_{ST,12} = \frac{[M_{12}]}{[M_{138}]}$. The $\Delta H$ and $\Delta S$ values extracted in this way can be used to back calculate a population distribution at an arbitrary temperature. These back-calculated free energy distributions (shown here for $\Delta G_{AV}$) were found to be in excellent agreement with that measured experimentally (C), confirming that the data can be well explained by considering the relative thermal stabilities of the individual oligomers. Although the dodecamer undergoes a large change in relative concentration over the temperature range studied, there is no evidence to suggest that the variation in size distribution is due to the formation of structurally distinct ‘activated’ complexes that radically alter the relative stabilities of the various complexes.
To determine a relationship for average m/z and average mass of the sHSP:client complexes we used two published data sets of charge state versus mass for a range of proteins and protein complexes. Overlaid is a theoretical prediction of the maximum charge state (dashed line), derived from the Rayleigh limit for droplet fission, \( Z_{\text{MAX}} = 0.0778 \sqrt{M} \). From the experimental data we can obtain a line of best fit (Solid Line) for the data with a relationship for the average charge state: \( Z_{\text{AV}} = 0.0467 M^{0.533} \). Therefore, by letting the m/z value of a peak in the mass spectrum be given by \( T \), and with the simplification that the mass of the charge-giving protons is insignificant compared to the mass of the protein complexes: \( M_{\text{AV}} = 21.413 M^{0.467} \) and therefore: \( T_{\text{AV}} = 0.00140 \). Based on these relationships, we would expect a hypothetical [HSP18.1]_{12} [Luc] complex to be centered on the 37+ charge state, with the principal five charge states therefore located between 7,094 and 7,905 m/z (39+ to 35+). To calculate \( M_{\text{AV}} \) for the sHSP:client complexes, the broad area of signal corresponding to complexes was integrated, and \( T_{\text{AV}} \) was taken to be the weighted median m/z value.

**Fig. S5.** SEC and SDS-PAGE of HSP18.1:Luc complexes. We formed complexes between HSP18.1 and Luc by incubating them at 42 °C for 10 min, at two different ratios, and analyzed them by means of SEC (A). At a 1:1 ratio (HSP18.1 dodecamer to Luc monomer), a broad peak centered on 10.5 mL, a fraction of which was used for MS analysis (Fig. S8), and a narrower peak at 13.7 mL were observed. SDS-PAGE, using pre-cut gels and the SeeBlue Plus 2 marker (both Invitrogen), of these peaks (B), show the former to contain both HSP18.1 and Luc, and the latter solely sHSP. Increasing the ratio of HSP18.1 to Luc results in a narrower complex peak and shifts it to longer elution times, implying a narrower distribution of lower average mass likely more amenable to MS analysis.
The general mechanism of dissociation of protein complexes upon collisional activation is the loss of highly charged monomers from the parent oligomers (6). Moreover, multiple subunits can be removed, in a sequential manner, depending on the amount of activation (1). Performing tandem-MS of the peak at 8,950 m/z, as in Fig. 3, results in monomers at low m/z, and two distinct regions of signal at high m/z, centered at approximately 14,000 m/z, and approximately 22,000 m/z, respectively. At an acceleration voltage into the collision cell of 75 V only the former is populated. As the voltage is increased the latter region becomes progressively more dominant, such that at 200 V most of the signal resides therein. This shows that these regions therefore correspond to oligomers stripped of one and two monomers, respectively.
Assignment of HSP18.1:Luc complexes. To assign the masses for complexes we measured in our tandem-MS spectra to particular combinations of HSP18.1 and Luc we constructed a matrix of theoretical masses based on the sequences of the individual proteins. Each measured mass was then compared to all possible combinations, and that with the lowest difference was taken to be the correct assignment. For example, from the spectrum shown in Fig. 3B, we obtained a mass from a charge state series of 402,578 Da. Comparing this with our theoretical matrix results in one possible combination, [HSP18.1]_x[Luc]_y, of much better correspondence than all others (A). The same procedure for other masses obtained from Fig. 3B, 366,591 Da and 384,584 Da, results in similarly unambiguous assignment (B). Common to all spectra of protein assemblies, a small discrepancy between measured and theoretical masses remains, due to the presence of residual solvent molecules and buffer ions (7).
Fig. S8. Complexes formed at different ratios of HSP18.1 and Luc. We selected a number of regions of the MS spectra obtained for the complexes for tandem-MS interrogation. For a 1:0.1 ratio, upper panel, four separate isolations were performed and combined to enable a reconstruction of the overall distribution. For the 11.5–12.3 mL fraction, from incubation at a 1:1 ratio (Lower, Fig. S4), additional isolations up to approximately 12,000 m/z were performed.

Fig. S9. Characterization of complexes between HSP18.1 and citrate synthase. Complexes were formed between HSP18.1 and citrate synthase (Sigma, Cat C5260-200UN) by incubation at a 1:0.25 molar ratio (dodecamer:dimer) at 45 °C for 60 min. A nES mass spectrum of the resultant complexes shows a broad area of signal from 7,000 to 14,000 m/z, consistent with a polydisperse ensemble of species (A) This mirrors what we observed with luciferase as client (Fig. 2A, Fig. 3A, Fig. S10). Selection and dissociation of the complexes at 8,900 m/z (B), allows the identification of different stoichiometries. In this spectrum we are able to unambiguously identify doubly stripped oligomers comprised of two citrate synthase subunits bound to between 15 and 18 subunits of HSP18.1. These experiments demonstrate that the polydisperse nature of client binding to HSP18.1 is not limited to Luc, but rather appears a general feature of this sHSP’s chaperone function.
Fig. S10. Extrapolation to obtain full distribution of complexes. To obtain an overall distribution for the complexes formed at a 1:1 ratio (Fig. S5C) we combined our tandem-MS results with the SEC profile. We derived a relationship between the number of HSP18.1 and Luc subunits from tandem-MS (A), and hence the mass dependence of the number of HSP18.1 (circles) and Luc (Triangles) subunits (B). From this we could estimate the relationship between mass and extinction coefficient (C). From the overall SEC profile (Solid Line) at a 1:1 ratio (D), the contribution of dodecamer (Dashed Line) was removed. By calibration using standard proteins (E), the SEC trace was converted to a mass axis, and, from the relationship in C, into a concentration scale (F). Gaussian distributions for the different numbers of HSP18.1 at each bound state of Luc, as in Fig. S5C, and of form $y = ae^{-(x-x_0)^2/(2b^2)}$ were constructed using the trends in mean number of HSP18.1 per Luc (parameter $x_0$) (A); peak width (parameter $b$) (G); and intensity (parameter $a$) (H), obtained by considering $F$ in relation to $B$. In panels A, B, and G empty symbols correspond to the tandem-MS data, and filled symbols to extrapolation. Combining the different Gaussian distributions results in the quantitative three-dimensional distribution map of different complexes (Fig. S5C).