Mechanism of Chaperone Function in Small Heat Shock Proteins

DISSOCIATION OF THE HSP27 OLIGOMER IS REQUIRED FOR RECOGNITION AND BINDING OF DESTABILIZED T4 LYSOZYMEx

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Mammalian small heat shock proteins (sHSP) form polydisperse and dynamic oligomers that undergo equilibrium subunit exchange. Current models of their chaperone activity hypothesize that recognition and binding of protein non-native states involve changes in the oligomeric state. The equivalent thermodynamic representation is a set of three coupled equilibria that includes the sHSP oligomeric equilibrium, the substrate folding equilibrium, and the equilibrium binding between the sHSP and the substrate non-native states. To test this hypothesis and define the binding-competent oligomeric state of human Hsp27, we have perturbed the two former equilibria and quantitatively determined the consequences on binding. The substrate is a set of T4 lysozyme (T4L) mutants that bind under conditions that favor the folded state over the unfolded state by 102–104-fold. The concentration-dependent oligomer equilibrium of Hsp27 was perturbed by mutations that alter the relative stability of two major oligomeric states including phosphorylation-mimicking mutations that result in the dissociation to a small multimer over a wide range of concentrations. Correlation of binding isotherms with size exclusion chromatography analysis of the Hsp27 oligomer equilibrium demonstrates that the multimer is the binding-competent state. Binding occurs through two modes, each characterized by different affinity and number of binding sites, and results in T4L:Hsp27 complexes of different hydrodynamic properties. Mutants of the Hsp27 phosphorylation mimic that reverse the reduction in oligomer size also reduce the extent of T4L binding. Taken together, these results suggest a central role for the oligomeric equilibrium in regulating the chaperone activity of sHSP. The mutants identify sequence features important for modulating this equilibrium.

Members of the small heat shock protein (sHSP) superfamily (1) are ubiquitous molecular chaperones that participate in physiological processes underlying stress tolerance (2), longevity and aging (3), and apoptosis (4, 5). Of the 10 sHSP identified in humans, αA- and αB-crystallin are resident lens proteins that maintain its optical properties (6, 7), whereas αB-crystallin and Hsp27 are abundant in muscle tissues where they protect against stressful conditions such as ischemia (8, 9). sHSP have molecular masses of between 12 and 40 kDa/subunit and are characterized by the presence of a conserved region in the C-terminal part of their sequences referred to as the α-crystallin domain (10). Invariably, sHSP form oligomeric structures of 4–40 subunits that recognize and bind non-native protein states in a process that has two distinguishing characteristics. First, sHSP have a remarkably higher capacity than any other chaperones, binding substrates in some cases with a 1 to 1 stoichiometry (7, 11–13). Second, recognition and binding do not involve the hydrolysis of ATP (1).

Associated with these functional characteristics is a unique molecular architecture (14–18). The emerging model is a polarized particle outlined by a solvent-exposed outer shell consisting of the conserved α-crystallin domain whereas the variable and predominantly hydrophobic N-terminal domains are clustered in the core. The scaffold is flexible and amenable to a wide range of dynamic behaviors. Not only are there wide variations in the number of subunits and the symmetry of their packing between sHSP from across the evolutionary spectrum, but also the oligomers have substantially different degrees of order and dispersity (19–22).

The need for such architecture is now better understood as a result of mechanistic studies of sHSP chaperone activity. Mammalian sHSP have sequence and structural elements that allow them to bind various aggregation-prone, non-native states of target proteins. Substrate recognition and binding are modulated by temperature and phosphorylation, thus providing a control mechanism for the chaperone activity (11–13, 23–27). The prevalent model links the regulation of binding to changes in the oligomeric structure possibly through subunit exchange (13, 21, 28, 29). This results in either a change in accessibility of the binding elements or a transition to a binding-competent conformation.

Little is known about the mechanism by which the change in the oligomeric state is controlled at the sequence level. Temperature activation of substrate-protein binding occurs below the melting temperature of the oligomer and parallels increases in subunit exchange rates (20, 21). This suggests that the dissociation of the oligomers is mediated by disruption of relatively weak subunit contacts. Site-directed truncation studies of mammalian sHSP indicate that interactions between the N-terminal domains of subunits may be responsible for the global assembly of the oligomer from smaller multimeric units (16, 20). Phosphorylation of residues in the N-terminal domain destabilizes the quaternary structure as illustrated by the dissociation of Hsp27 (30).

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Hsp27 Binding to T4L Requires Oligomer Dissociation

revealed that α-crystallins can bind their substrates in two different modes each characterized by different affinity and number of binding sites (12, 13, 27). Patterns of fluorescence quenching of a bimane probe attached to the model substrate, T4L, are consistent with a distinct conformation of bound T4L in each mode (27). Quantitative comparison of binding isotherms reveals activation of the α-crystallins in response to increased temperature, pH, and phosphorylation. The parallel changes in the binding characteristics and oligomeric structure led to the hypothesis that the chaperone activity requires the dissociation of the oligomer (28, 31, 32). In this model, the predominant native oligomer is a storage state that is destabilized by stress signals such as increased temperature in lower organisms and phosphorylation in mammalian cells and hence provides a switch to turn on these molecular chaperones.

Human Hsp27 is a unique model system for testing this hypothesis and exploring the sequence determinants of quaternary structure variability and dynamics. Unlike its homologs αA- and αB-crystallin, Hsp27 dissociates into a discrete small oligomer at low concentration and/or high temperature in a process that can be conveniently monitored by size exclusion chromatography (SEC) (30, 33, 34). In this paper, we take advantage of this experimental process to design mutants of Hsp27 that alter the population of either oligomeric state. The changes in the oligomer equilibrium are then correlated with the changes in the binding characteristics to the model substrate T4L. Critical in highlighting the differences between Hsp27 variants is the binding of T4L under conditions where its native state predominates. The results demonstrate a thermodynamic coupling between the equilibria that corresponds to its native state predominates. The results also identify sequence motifs involved in driving oligomer dissociation.

**EXPERIMENTAL PROCEDURES**

**Cloning and Site-directed Mutagenesis**—The detailed description of the cloning and site-directed mutagenesis of T4L were previously reported (13, 35). Mutants of Hsp27 were constructed using a modified megaprimer PCR method. The PCR fragments were then subcloned into the original pET-20b vector and transformed into E. coli competent cells (16). Plasmid DNA purified from the transformed cells was then sequenced to confirm the presence of the mutant and the absence of unwanted changes. In this paper, single-site mutants are named by specifying the original residue, the position number of the residue, and the new residue.

**Protein Expression, Purification, and Labeling**—Briefly, T4L mutants were expressed in the E. coli strain K38 using permissive induction temperatures as described previously (13). Sequential cation exchange and size exclusion chromatographies were used to purify the mutants to apparent homogeneity. Following elution from the cation exchange column, the mutants were incubated with a 10-fold molar excess of monobromobimane in a pH 7.6 buffered solution (36). The reaction (Scheme 1) was allowed to proceed for 2 h at room temperature and then overnight at 4 °C. The reaction mixture was then purified by SEC using a Superdex 75 column equilibrated with the appropriate buffer. Different pH values were obtained by varying the molar ratios of Mes and Tris while maintaining the total ionic strength constant at 65 mM. The labeling efficiency was determined by comparing the absorption at 280 and 380 nm. Consistently a 280/380 ratio of 10:1 was obtained. This ratio indicates stoichiometric labeling of the introduced cysteine. The protein concentrations were determined using an extinction coefficient of 1.228 cm²·mg⁻¹ · cm⁻¹ for T4L and 1.78 cm²·mg⁻¹ · cm⁻¹ for Hsp27 (Scheme 1).

![Scheme 1](image)

**RESULTS**

**Binding Characteristics of Hsp27 and Its Phosphorylation Mimic Hsp27-D3**—Table I reports the ΔG_unf values of the T4L mutants used in this study. Details of the nature, rationale, and the structural consequences of the mutations were described previously (13). In summary, the unique properties of this substrate model system include an apparent two-state folding equilibrium, wild type-like structure of the mutants, and progressively lower free energies of unfolding (ΔG_unf) in the 3–8 kcal/mol range at 23 °C. Except for T4L-D70N, the ΔG_unf of the mutants decreases with increasing pH, which provides another control parameter (13). Each T4L mutant has an introduced cysteine on the surface site 151 to allow the attachment of a fluorescent or paramagnetic re-
porter group. Changes in the spectral characteristics of the reporter allow the detection of the substrate-chaperone complex. The interpretation and origin of these changes were previously discussed (13, 27).

Lens α-crystallins, sHSP with substantial homology to Hsp27, bind T4L mutants under conditions where the folding equilibrium constants are in the 10^4–10^8 range (12, 13). Progressively destabilized T4L mutants have higher extent of binding reflecting the energy balance between association with the chaperone and refolding from the non-native state recognized by the chaperone (13, 27). Similarly, Hsp27 binding to T4L mutants correlates with the ΔG\text{calc} of the latter. At 37 °C, the only mutant that binds to a significant extent is the least stable, T4L-L99A/A130S. Fig. 1a shows the binding isotherm along with a nonlinear least squares fit to a single mode binding model. Binding occurs with a characteristic number of binding sites n = 1 corresponding to one T4L molecule/Hsp27 subunit (Table II). In the bound state, the emission intensity of the bimane is substantially quenched relative to its emission in the T4L native state. A similar level of quenching following binding to α-crystallins was shown from the extensively unfolded conformation of the bound T4L (27).

Phosphorylation of Hsp27, mimicked by the substitution of aspartates for the native serines at sites 15, 78, and 82, results in a substantial increase in the extent of binding as shown in Fig. 1b (a and c). The apparent affinity of this Hsp27 variant, hereafter referred to as Hsp27-D3, to T4L-L99A/A130S is 4-fold larger than that of the WT (Table II). The binding isotherm was constructed at a fixed T4L concentration of 3 μM because at a 8 μM concentration mixed T4L-Hsp27-D3 precipitates were observed at low molar ratios of Hsp27 to T4L. For a marginally stable mutant such as T4L-L99A/A130S, Hsp27-D3 becomes saturated with T4L in this range of molar ratios leading to a loss of solubility. The increased extent of binding by Hsp27-D3 is also demonstrated by the binding of the more stable T4L mutant D70N shown in Fig. 1c. Analysis of the binding isotherm reveals that the change in the binding characteristics in the D3 variant is not only manifested in the apparent dissociation constant but also by a reduced number of binding sites compared with T4L-L99A/A130S (Table II). This suggests that Hsp27 can bind its substrate in a high or low affinity mode each with a different n. The observed mode of binding depends on the phosphorylation state of Hsp27 as well as the ΔG\text{calc} of T4L.

Unlike α-crystallin, high affinity and low affinity binding by Hsp27 result in similar levels of bimane quenching and hardly any blue shift of the emission λ\text{max}. In the context of T4L, bimane undergoes a sequence-dependent quenching upon global unfolding of the protein (27). Although the mechanism underlying this effect has not been elucidated, it provides a convenient tool to probe the extent of unfolding of T4L in the bound state. Attachment of the bimane at site 116 in the L99A background concomitantly reduces the level of quenching upon denaturant unfolding and upon binding to the αB-crystallin phosphorylation mimic, αB-crystallin S19D/S45D/S59D (27). However, for binding to Hsp27 the quenching is similar whether the bimane is attached at sites 151 or 116 (data not shown), suggesting additional modes of quenching in the bound state such as proximity to tryptophan and tyrosine residues of Hsp27 (38).

To further explore the interplay between the two binding modes, we characterized the binding of T4L-L99A to Hsp27-D3 at 37 °C, pH 7.2. The stability of this mutant allows the construction of binding isotherms over a wide range of spectrally accessible concentrations. Table III reports the parameters obtained from nonlinear least squares fits of two isotherms constructed at fixed T4L concentrations of 3 and 6 μM. The transition of n from 0.3 to 0.9 indicates that the mode of binding is not only dependent on the stability of the T4L mutant but is also determined by the range of concentrations over which Hsp27-D3 is varied. Concentration-induced changes in n are observed when the fixed concentration of T4L-D70N in Fig. 1 is raised from 8 to 20 μM and in the room temperature binding isotherms of Hsp27-D3 to T4L-L99A/A130S at 10 and 30 μM as reported in Table III. Because the fluorescence of T4L bound through both modes is similar, the coexistence of the two modes is not manifested by the biphasic isotherms as in the case of

![Figure 1](http://www.jbc.org/)

**TABLE II**

| T4L mutant | Hsp27 | K_D | n |
|------------|-------|-----|---|
| L99A/A130S (8 μM) | WT | 0.17 ± 0.04 | 1.1 ± 0.02 |
| L99A/A130S (3 μM) | D3 | 0.04 ± 0.02 | 1.1 ± 0.04 |
| D70N (8 μM) | D3 | 0.20 ± 0.08 | 0.2 ± 0.01 |
binding to α-crystallin. In the transition region, two-mode binding is reflected in a continuous range of $n$ values.

The Mode and Extent of T4L Binding Correlate with the Predominant Oligomeric State—Analysis of Hsp27 molecular mass by SEC at low concentration reveals an equilibrium between at least two oligomeric states characterized by substantially different retention times as shown in Fig. 2a. For the highly homologous mouse Hsp25, a concentration-dependent equilibrium between a hexadecamer and a tetramer was reported (33). The average molecular mass of the larger oligomer of human Hsp27 was determined by SEC to be 530 kDa (39). Higher concentrations shift the equilibrium toward the large oligomer, whereas phosphorylation, mimicked by the serine to aspartate substitutions (Fig. 2b), favors the smaller species by shifting the window of the transition to higher concentrations. For instance, at 1 mg/ml Hsp27 is primarily a large oligomer, whereas Hsp27-D3 injected at 5 mg/ml migrates at the retention time of the smaller oligomer, and even the SEC chromatogram of concentrations as high as 28 mg/ml of Hsp27-D3 reveals only partial reassembly. Enhancement in the equilibrium population of the multimer is also observed in SEC carried out at 37 °C (data not shown). The size of the Hsp27-D3 multimer was reported to be a tetramer based on SEC (30).

However, the estimate was based on a calibration curve and thus may be influenced by differences in the hydrodynamic properties of Hsp27-D3 relative to the proteins used as standards. This is particularly important if dissociation results in changes in the structure and shape of the Hsp27 subunits. Nevertheless, these estimates provide an approximate range for the mass of the multimer that clearly distinguishes it from the larger species. Taken together, the data reported in Table II and the SEC of Fig. 2 demonstrate that enhanced population of the multimer, regardless of its exact molecular mass, leads to binding with a higher apparent affinity.

Single and double aspartate variants of Hsp27 have also been reported to enhance dissociation of Hsp27 although to a smaller extent than the D3 substitutions (30). We have tested the binding characteristics of the S15D and S78D/S82D mutants. In both cases, the substitutions result in enhancement of T4L binding relative to WT Hsp27. The enhancement correlates with the level of substitution: Hsp27-S78D/S82D binds T4L to a larger extent than Hsp27-S15D under the same conditions (data not shown).

Perturbation of the Hsp27 Oligomer Equilibrium Affects the Extent of T4L Binding—If the dissociation to smaller oligomer is at the origin of the enhancement in the extent of binding of Hsp27-D3, then conversely an increase in the population of the large oligomer will be accompanied by a shift to the low affinity, $n = 1$, mode and will be manifested by a reduction of bound T4L. A direct approach to test this model is to construct variants of Hsp27 or Hsp27-D3 that change the relative stability of the two predominant oligomeric states. To design such mutants, we surveyed the N-terminal domain of Hsp27 for highly conserved charged residues. The rationale is that phosphorylation sites in both Hsp27 and αB-crystallin occur in the N-terminal domain.

Table III

| T4L mutant | Temperature °C | pH | T4L concentration | $K_D$ | $n$ |
|------------|----------------|----|-------------------|------|----|
| L99A       | 37             | 7.2 | 3                 | 0.04 ± 0.03 | 0.3 ± 0.01 |
| L99A       | 37             | 7.2 | 6                 | 0.76 ± 0.17 | 0.85 ± 0.05 |
| L99A/A130S | 23             | 8.0 | 10                | 0.7 ± 0.3   | 0.3 ± 0.1   |
| L99A/A130S | 23             | 8.0 | 30                | 1.52 ± 0.55 | 0.87 ± 0.04 |
| D70N       | 37             | 7.2 | 20                | 1.11 ± 0.24 | 0.55 ± 0.02 |

Structural studies of Hsp16.5 and Hsp16.9 revealed that the N-terminal regions of these proteins are relatively solvent-inaccessible (14, 15). In such a low dielectric medium, unpaired charges are likely to destabilize the oligomer.

One of the charges identified is an acidic residue, Glu$^{41}$, which is also conserved in lens α-crystallin. Fig. 3a shows that substitution of this residue with a cysteine in the Hsp27-WT background stabilizes the large oligomer. More importantly, the single substitution reverses the effects of the three serine to...
aspartate substitutions. Fig. 3c shows that at 1 mg/ml the mutant Hsp27-D3-E41C has a retention time similar to the large oligomer in the WT background (a) and the D3 background (b). c, both Hsp27-D3-E41C and Hsp27-D3-Del undergo the concentration-dependent dissociation. Analysis was carried out under the conditions of Fig. 2. The traces of Hsp27 and Hsp27-D3 are included for reference. RT, room temperature.

Another design strategy is to identify sequence elements associated with the differences in the dynamic behavior of the Hsp27 and α-crystallin oligomers. Although both are polydisperse and undergo subunit exchange, Hsp27 is unique in its propensity to dissociate into discrete multimers. In addition to sequence divergence in the N-terminal domain, Hsp27 differs from α-crystallins by a peptide insertion near the N- and C-terminal domain junction. The peptide sequence, which includes residues 58–70, is relatively redundant with numerous alanine and proline residues. We reasoned that this flexible peptide may be associated with the distinct dynamic characteristics of Hsp27. The absence of this insertion in the α-crystallins implies that its deletion would be tolerated. Consistent with this conclusion, an Hsp27 variant, Hsp27-Del lacking this sequence expresses in the water-soluble fraction. Similar to the E41C substitution, the deletion of this peptide in both the Hsp27 and Hsp27-D3 backgrounds shifts the equilibrium toward the large oligomer (Fig. 3c). Fig. 4 shows that the mutation-induced changes in the oligomer equilibrium occur without deleterious effects on the protein structure.
secondary and tertiary structures. Far UV CD spectra of the mutants have a minimum at 217 nm, indicating predominant \( \beta \)-sheet structure. Furthermore, the shape of the near UV spectra is consistent with a packed structure in the vicinity of the aromatic residues. Pronounced changes in the shape of the far UV spectra for Hsp27-D3 and Hsp27-D3-Del may reflect limited structural alterations in the multimer, which is the dominant state of these mutants at 0.2 mg/ml. Similarly, the observed minor differences in the near UV spectra may arise from the changes in the environments of some of the aromatic residues because of the dissociation of the oligomeric structure in the D3 variants.

For all variants, the shift in the equilibrium toward the large oligomer (Fig. 5) correlates with reduced extents of T4L binding (Fig. 5) even in the Hsp27 background where the large oligomer is already the predominant state. The T4L concentration, pH, and temperature in Fig. 5 were optimized to maximize binding to either Hsp27 or Hsp27-D3. For instance, at pH 7.2 Hsp27 binding to T4L-L99A is marginal, whereas it is significantly enhanced at pH 8.0 to allow for the construction of a binding isotherm. The reduced binding to the E41C and Del variants hinders a quantitative comparison of the isotherms at the T4L concentration utilized.

The differences in the binding characteristic of Hsp27, Hsp27-D3, and their E41C and Del mutants are reduced when isotherms to the more destabilized mutants such as T4L-L99A/ A130S are compared (data not shown). This suggests that small \( \Delta G_{\text{dest}} \) of the substrate, such as those achieved in aggregation-based assays, can in a coupled equilibrium mask the subtle differences in the \( \Delta G \) of the Hsp27 dissociation caused by the mutations. Thus, the choice of mutants, pH levels, and concentrations of T4L are critical in allowing the observation of the different binding parameters of Hsp27 and Hsp27-D3 and their respective variants.

**pH-induced Shifts of the Hsp27 Oligomer Equilibrium**—A significant effect of pH on the extent of T4L binding to \( \alpha \)-crystallins was previously reported (12, 13). That this effect was observed using T4L-D70N, a mutant characterized by pH-independent \( \Delta G_{\text{dest}} \) (40), led to the conclusion that it originates from a change in the binding characteristics of the chaperones. However, because \( \alpha \)-crystallins oligomer equilibrium is not manifested by distinct peaks on SEC, the effect of the pH change on this equilibrium was deduced from subtle shifts in the average retention time of the size exclusion envelope (39).

For Hsp27 and its variants a reduction in pH results in a shift in the oligomer equilibrium toward the large oligomer. The Hsp27-WT SEC chromatogram at pH 6.5 consists of a single peak even at concentration as low as 0.01 mg/ml (data not shown). Perhaps most illustrative of the destabilizing effect of lower pH on the multimer is the concomitant changes in the populations of the two oligomeric states in the equilibrium of Hsp27-D3-Del (Fig. 6a). Comparison of the binding isotherms at three different pH values, shown in Fig. 6b, demonstrates that the shift in the equilibrium is accompanied by a reduction in the extent of binding to T4L. For the same molar ratio of Hsp27-D3-Del to T4L, a significant increase in the fraction of bound T4L is observed at higher pH. Based on the lack of significant changes in the \( \Delta G_{\text{dest}} \) of T4L (40), we conclude that the increased binding is the result of a shift in the equilibrium between the two predominant oligomeric states of the chaperone in solution.

**The Two Binding Modes Result in Hsp27-T4L Complexes of Different Sizes**—The data presented above establish a direct link between the tendency to dissociate and the extent of binding and suggest that the multimer is the active oligomeric state. Given that the transition between the two modes results in a 4-fold increase in the binding capacity, changes in the mass and/or size of the Hsp27-T4L complexes are expected depending on the predominant mode of binding.

To test this prediction, we characterized the hydrodynamic properties of the Hsp27-T4L complexes obtained in the two binding modes by comparing the retention time of the complexes using SEC. The migration of the complex relative to Hsp27 and T4L can be conveniently monitored by simultaneous detection of absorption at 280 nm and bimane fluorescence at 468 nm as described previously (41). The retention times, reported in Table IV, were characterized under conditions that promote either high (\( n = 0.25 \)) or low (\( n = 1 \))

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**Figure 5.** Changes in the binding characteristics as a consequence of the E41C substitution and the peptide deletion. *a,* binding isotherms of Hsp27 variants to T4L-L99A. *b,* binding isotherms of Hsp27-D3 variants to T4L-D70N. In both panels the T4L concentration is fixed at 6 \( \mu \)M, whereas Hsp27 concentration is varied to achieve the required molar ratio.
affinity binding, and the concentrations used directly correspond to those of the binding isotherms. For reference, Table IV lists the retention times of Hsp27 and Hsp27-D3. For low affinity binding by Hsp27, the bimane fluorescence was almost exclusively detected in a peak that runs in the void volume. In contrast, high affinity binding by Hsp27-D3 resulted in a fluorescence peak characterized by a retention time closer to the large oligomer than the multimer. Notably, no evidence of a significant population of a multimer-T4L intermediate was observed. In addition, binding by Hsp27-D3 under conditions that yield \( n = 1 \) shifted a significant amount of fluorescence to a peak that eluted in the void volume (Table IV). Taken together, these results demonstrate that binding reassembles the multimers into a large complex. Furthermore, they establish the physical significance of the \( n \) values obtained from the binding isotherms.

**DISCUSSION**

This paper expands our investigation of the mechanism by which Hsp27 and mammalian sHSP “sense” protein stability to include the role of oligomer disassembly in substrate recognition and binding. Mammalian sHSP form oligomers of unique conformational and dynamic properties (6, 19, 22, 33, 42). Based on previous results (6, 28–30, 43), we propose that the multiple oligomeric states are in an equilibrium whose constant can be manipulated by temperature and phosphorylation at specific residues. Multiple lines of indirect evidence support a role of oligomer heterogeneity and dynamics in chaperone efficiency defined as the ability of sHSP to suppress the aggregation of a particular substrate.

From a thermodynamic perspective, the postulated exposure of the substrate binding regions through the equilibrium dissociation of the native oligomer implies that the interconversion of the oligomer between multiple states is coupled to the folding equilibrium of the substrate. A prediction of this model is that the latter equilibrium will be shifted by changes in the equilibrium constant of the Hsp27 transition and vice versa. Therefore, there are two critical cornerstones for the experimental paradigm of this paper. First, the ability to shift the oligomer equilibrium of Hsp27 without changes in other structural and/or physical/chemical properties. This is achieved via rational, site-directed mutagenesis based on sequence analysis and an emerging structural model of sHSP quaternary structure. Second, the use of a model substrate that is predominantly in the folded state. If the unfolded state predominates, the overall free energy of the coupled system primarily reflects this equilibrium, and thus subtle changes in the Hsp27 equilibrium are masked. This latter aspect may explain the reported results in the literature that phosphorylation of Hsp27 and \( \alpha \)-B-crystallin did not significantly affect chaperone efficiency (30, 43). Efficiency assays are carried out under conditions that trigger global unfolding of the substrate essentially resulting in a negative \( \Delta G_{\text{unf}} \).

**Oligomer Dissociation Mediates a Conserved Mechanism of Substrate Recognition and Binding**—Both \( \alpha \)-Crystallins and Hsp27 differentially interact with T4L mutants having similar structures in the native state but different free energy of unfolding. Under equilibrium, proteins undergo structural fluctuations that result in the transition from the predominant solu-

![Fig. 6. Origin of the pH-induced activation of binding. a, SEC demonstrates that higher pH enhances dissociation. All three traces were obtained by injecting the same amount of Hsp27-D3-Del. b, changes in the extent of binding of Hsp27-D3-Del to T4L-D70N at different pH. In all three isotherms, a constant concentration of T4L (6 \( \mu \text{M} \)) was used.](http://www.jbc.org/)

**Table IV**

| T4L mutant   | T4L concentration | Hsp27 Molar ratio (T4L:Hsp27) | \( n \) | \( R_t \) min |
|--------------|-------------------|------------------------------|------|-------------|
|              | \( \mu \text{M} \) |                              |      |             |
| WT           |                   | D3                           | 1:10 | 0.2         | 25.4 |
| D3           |                   | D3                           | 1:8  | 1           | 31.4 |
| D70N         | 8                  | D3                           | 1:10 | 0.9         | 24   |
| L99A/A130S   | 3                  | WT                           | 1:1  | 1           | 14.8 (void volume) |
| L99A/A130S   | 8                  | D3                           | 1:8  | 1           | 14.9 (void volume) |
| L99A         | 3                  | D3                           | 1:4  | 0.3         | 23   |
| L99A         | 6                  | D3                           | 1:4  | 1           | 15.3 (void volume) |
tion state, the folded state, to high energy forms characterized by various degrees of unfolding (44–46). In principle, molecular chaperones should recognize and bind partially unfolded states irrespective of whether they are kinetic by-products of folding or populated under equilibrium. The formation of stable complexes depends on the balance between free energies of binding and refolding, hence the observed dependence of the extent of binding on \( \Delta G_{\text{out}} \) of the substrate.

The enhanced binding of T4L to the phosphorylated mimic of Hsp27 also parallels that observed for \( \alphaB \)-crystallin (12). The novel result of this paper is that the enhancement of Hsp27 binding to T4L is correlated with oligomer dissociation, whereas changes in the oligomer equilibrium of \( \alphaB \)-crystallin were manifested as small shifts in the retention time of the broad envelope on SEC (12). Effectively, the serine to aspartate substitutions shift the concentration dependence of Hsp27 association to higher concentrations. For both Hsp27 and \( \alphaB \)-crystallins, the phosphorylation-induced increase in the extent of binding occurs through a change in the binding mode, although we did not observe the biphasic binding isotherms characteristic of the two-mode binding of T4L by \( \alphaB \)-crystallin S19D/S45D/S59D. This is due to the similar emission properties of the bimane emission in the two binding modes of Hsp27 (Fig. 1).

That the two modes coexist is reflected in values of \( n \) that span the range from 0.25 to 1 (Table III).

The central conclusion of this paper is that the dissociation of the Hsp27 oligomer is required for substrate binding. This conclusion is not only supported by the binding enhancement of Hsp27-D3 but also by the binding characteristics of mutants that partly reverse the effect of the D3 substitutions. In each case, the decrease in the intrinsic preference for the multimer is accompanied by a reduction in the extent of T4L binding under similar conditions of pH, concentration, and temperature. This effect is pronounced for the more stable T4L mutants, whereas the more destabilized ones tend to reduce the differences between the Hsp27 and Hsp27-D3 variants as predicted by a coupled equilibrium model. The interplay between the two oligomeric states also provides the basis for the pH-induced activation of binding that was previously reported for \( \alphaB \)-crystallins (13, 27). Lower pH that reduces the fraction of T4L-70N bound over the entire range of Hsp27-D3-Del concentrations concomitantly favors the large oligomer (Fig. 6).

Given the dependence of the oligomeric state on concentration, binding isotherms should ideally be constructed at a fixed concentration of Hsp27. In this type of experiment, the emission intensity of each T4L concentration is normalized against a control sample that does not include Hsp27 or its variants. However, we observed that over the necessary range of T4L concentrations, the bimane emission is not a linear function of concentration. At the high end, an inner filter effect reduces the excitation intensity, whereas at the lower end residual nonspecific interactions with the cuvettes resulted in deviations from linearity. Together, these two sources of errors resulted in significant uncertainties in the fit values of \( n \) and \( K_D \). It is noted that for the binding isotherms presented in Fig. 1, the concentration range of Hsp27-D3 does not result in reassembly to large oligomers (Fig. 2). Similarly, the range of concentrations in the binding isotherms of Hsp27 is above that corresponding to the transition region from multimer to large oligomer at pH 7.2.

The Two Modes of Binding Result in Distinct Hsp27-T4L Complexes—Although the multimer is the binding competent state, we did not detect a significant equilibrium level of this species in a complex with T4L. The lack of an intermediate suggests that substrate binding induces multimer reassembly into larger complexes. The size and the hydrodynamic properties of the resulting Hsp27-T4L complex are a consequence of the mode of binding and reflect the value of \( n \). Our observation of binding-induced reassembly is consistent with previous studies using Hsp26 (47), another sHSP that dissociates into a discrete multimer, and suggests that the mechanism of binding may be conserved in sHSP.

Thermodynamic model of Hsp27-T4L interaction—From a thermodynamic perspective, the chaperone substrate system is represented by three equilibria.

\[
N = I_1 = \ldots = I_n = U \quad (\text{Eq. 1})
\]

\[
L = p(M) \quad (\text{Eq. 2})
\]

\[
M + \frac{I_i}{U} = \frac{C_i}{C_u} \quad (\text{Eq. 3})
\]

Equation 1 is the folding equilibrium of the substrate where \( I_i \) indicates intermediate states, and \( N \) and \( U \) are the folded and unfolded states, respectively. Equation 2 represents the dissociation of Hsp27 from a large oligomer (\( L \)) to the multimer (\( M \)); \( p \) is an integer that accounts for the difference in the number of subunits between the two oligomeric states. Equation 3 describes the formation of Hsp27-T4L complexes \( C_i \) through \( C_u \).

In the equilibrium binding assay, only the concentrations of the substrate native state, \( N \), and the chaperone, Hsp27, are known. Therefore, the apparent dissociation constant measured from the binding isotherms reflects, in addition to the chaperone intrinsic binding constant (Equation 3), the dissociation constant of Hsp27 to the binding-competent state \( M \) (Equation 2), and the equilibrium constant that characterizes the equilibrium of T4L between the folded state and the intermediate(s) recognized by Hsp27 (Equation 1). The coupled equilibrium implies that binding can be driven by a \( \Delta G_{\text{out}} \) of Equation 1 that favors \( f \) such as the case for highly destabilized T4L mutants or by a large population of chaperone in binding-competent state(s), \( M \), such as the case for Hsp27-D3.

The contribution of each binding mode to an isotherm depends on the concentration of T4L relative to its \( K_D \). For instance, at low fixed concentrations of T4L relative to the \( K_D \) of the low affinity mode, high affinity binding dominates, whereas at high concentrations of T4L relative to both \( K_D \), the two modes of binding contribute. However, even in the latter case the binding isotherms appear monophasic because of the similar fluorescence properties of T4L bound to Hsp27 in either mode. When the concentration of T4L is in the intermediate range, the two-mode nature of binding is manifested by \( n \) values between 0.25 and 1 (Table III). Progressively destabilized mutants of T4L have smaller folding equilibrium constant (Equation 1) and thus higher affinities (i.e. lower apparent \( K_D \)). Consequently, the onset of two-mode binding occurs at lower concentrations for T4L-L99A than for T4L-D70N (Table III).

For the same T4L mutant, higher concentration promotes contribution by the \( n = 1 \) mode resulting in the concentration dependence of \( n \). Furthermore, binding at higher concentrations of T4L requires a higher concentration of Hsp27, which reduces the equilibrium population of the multimer and results in lower affinity (larger \( K_D \)). This conclusion is valid irrespective of the binding conditions as long as they are identical at the two concentrations compared and carried out with the same T4L mutant.

The coupling of equations 1 and 2 also implies that the presence of T4L is expected to shift the equilibrium of Hsp27 given that the two limiting oligomeric states have different binding affinities. Therefore, the profiles of Figs. 2 and 3, although reflecting the intrinsic tendency of Hsp27 and Hsp27-D3 variants for dissociation, are not representative of...
the relative populations in the presence of the substrate. Because of the binding-induced reassembly demonstrated by SEC, it is not possible to characterize these equilibria in the presence of T4L. Nevertheless, comparative analysis of the binding characteristics under similar conditions and in the presence of the same T4L mutant will reflect the preference ranking deduced from Figs. 2 and 3.

Conformation of Bound T4L—The presence of two modes of α-crystallin binding was interpreted as arising from the binding of T4L in at least two different conformations (12, 13, 27). We proposed that high affinity binding occurs to compact ex-

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Mechanism of Chaperone Function in Small Heat Shock Proteins: DISSOCIATION OF THE HSP27 OLIGOMER IS REQUIRED FOR RECOGNITION AND BINDING OF DESTABILIZED T4 LYSOZYME

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