Self-association and Chaperone Activity of Hsp27 Are Thermally Activated*

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The small heat shock protein 27 (Hsp27) is an oligomeric, molecular chaperone in vitro. This chaperone activity and other physiological roles attributed to Hsp27 have been reported to depend on the state of self-association. In the present work, we have used sedimentation velocity experiments to demonstrate that the self-association of Hsp27 is independent of pH and ionic strength but increases significantly as the temperature is increased from 10 to 40 °C. The largest oligomers formed at 10 °C are ~8–12 mer, whereas at 40 °C oligomers as large as 22–30 mer are observed. Similarly, the chaperone activity of Hsp27 as indicated by its ability to inhibit dithiothreitol-induced insulin aggregation also increases with increased temperature, with a particularly sharp increase in activity as temperature is increased from 34 to 43 °C. Similar studies with an Hsp27 variant that mimics the behavior of the phosphorylated protein establish that this protein has greatly diminished chaperone activity that responds minimally to increased temperature. We conclude that Hsp27 can exploit a large number of oligomerization states and that the range of oligomer size and the magnitude of chaperone activity increase significantly as temperature is increased over the range that is relevant to the physiological heat shock response.

Heat shock proteins (Hsps) are molecular chaperones that are constitutively expressed under physiological conditions and are highly up-regulated during cellular stress. The small heat shock proteins (sHsps) are a subclass of Hsps that are characterized by low monomeric molecular mass (9–40 kDa) and a conserved α-crystallin domain near the N terminus (1). Most sHsps can form large oligomers in vivo and in vitro and are known to bind to non-native proteins during cellular stress. Though the mechanism by which sHsps bind to unfolded polypeptides is unknown, there is evidence that each oligomer binds to several non-native proteins and that the binding and release of substrates requires neither binding nor hydrolysis of ATP (2). Release of unfolded proteins bound by sHsps alone is, in fact, highly inefficient though both protein release and refolding can be achieved with the assistance of larger heat shock proteins (e.g. Hsp70) in an ATP-dependent reaction (3). A variety of unfolded substrate proteins can be bound by small heat shock proteins, and the size and stoichiometry of the resulting complexes vary with the nature of the unfolded protein (4).

The size of the oligomers formed by small heat shock proteins varies considerably with the species studied and is often regulated by phosphorylation of specific seryl residues (5). Some sHsps have well defined oligomeric structures that do not seem to be in equilibrium with smaller or larger species. Hsp16.9 from wheat, for example, forms a stable dodecamer that has been characterized by crystallographic structure determination (6). Similarly, Hsp16.5 from a thermophilic bacterium forms a compact, crystallographically characterized structure of 24 monomers (7). On the other hand, Hsp33 exhibits unusual behavior in that the reduced protein is monomeric and the oxidized protein is a disulfide-linked dimer (at 25 °C) that forms tetramers or octamers at elevated temperature (8); a dimeric form of this protein with an exchanged domain has been characterized by crystallographic structure determination (9, 10). We note, however, that Hsp33 lacks an α-crystallin domain and thus is not a small heat shock protein by definition. In general, however, sHsps such as Hsp27 and α-crystallin have been reported to be more dynamic in solution insofar as they undergo continuous subunit exchange (11).

Human Hsp27 exhibits a number of biological functions in addition to chaperone activity. Specifically, Hsp27 interferes with caspase activation (12–14), modulates oxidative stress (15), and regulates the cytoskeleton (16). Moreover, functional properties of Hsp27 vary with the state of self-association of the protein. For example, Hsp27 binds to cytochrome c as a large, unphosphorylated oligomer to inhibit the intrinsic apoptotic pathway (13), whereas it binds to Daxx as a small, phosphorylated oligomer to inhibit the extrinsic apoptotic pathway (14).

We have previously shown that in solution wild-type human Hsp27 is a highly dynamic oligomer that self-associates to form species as large as 12 and 16 mer (20 mM Tris-HCl, pH 8.4, 100 mM NaCl, 20 °C). Oligomers of smaller size, probably tetramers, monomers, and dimers, were also observed. Different behavior was observed for the S15D/S78D/S82D triple variant of Hsp27, which has been proposed to mimic the properties of phosphorylated Hsp27 in vitro (17). This variant forms primarily monomers and dimers under solution conditions in which the wild-type protein exhibits significant formation of larger oligomers, but the triple variant also forms small amounts of larger oligomers comparable in size with those formed by the wild-type protein (18). Evidently, the presence of the charged residues at these specific sites greatly reduces oligomerization and favors formation of monomers and dimers.

We have now studied the dependence of the self-association properties of wild-type Hsp27 and the S15D/S78D/S82D triple variant (Fig. 1) on pH, ionic strength, and temperature. In addition, we have determined the dependence of the chaperone activity of both the wild-type and variant proteins on temperature. The results of this work establish a...
new functional dimension to Hsp27 that has direct implications for the mechanism by which this protein functions in the heat shock response.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Protein Purification**—The coding sequence of human Hsp27 was amplified from the plasmid pCI-NeoHSP27/human kindly provided by Prof. J. Landry (Centre de Recherche en Cancérologie de l'Université Laval, Quebec). The gene was modified and cloned in pET30 vector (Novagen) as previously reported (18). This same vector was also used to clone the Hsp27 point variant S15D/S78D/S82D (Fig. 1). Protein expression and purification were achieved by the protocol previously published (18).

**Sedimentation Velocity Analyses**—The solution densities and partial specific volumes at all temperatures were calculated with the program SEDNTERP (19). The monomer molecular weights and molar absorptivities were calculated from the amino acid sequences with the program ProtParam provided by the ExPasy web site (20).

Sedimentation velocity experiments were conducted at 10, 20, 30, or 40 °C with a Beckman Optima XL-I analytical ultracentrifuge equipped with both absorbance and interference optics. Protein samples were diluted to the final concentration and dialyzed overnight against 1.5 liters of the appropriate buffer prior to analysis. Most experiments were performed with protein prepared in Tris-HCl buffer (20 mM, pH 8.4, 100 mM NaCl). Additional experiments of the wild-type protein were performed with protein prepared in sodium phosphate buffer (20 mM, pH 7.4, 100 mM NaCl). Standard 12-mm aluminum double-sector centripieces were filled with protein solution (400–450 μl), and the dialysis buffer was used in the reference cell. Before each run, cells were thermally equilibrated in the centrifuge for at least 1 and up to 8 h after the instrument had reached the final temperature under vacuum. Sedimentation velocity experiments were performed as a function of pH from 8.1 to 8.4. The results obtained with both the wild-type protein (0.6 mg/ml) and the triple variant (0.6 mg/ml) were normalized by dividing the S_{av} values to the density and viscosity at 20 °C. This analysis permits the direct comparison of S_{av} values obtained at various temperatures with each other. For wild-type Hsp27, the runs used at each temperature were as follows: 10 °C (1–30 S), 30 °C (1–50 S), and 40 °C (1–50 S). The S_{av} for samples with low concentrations of the triple variant were obtained by integrating the c(s) curves up to 5.5 S. To obtain c(s) curves corrected to standard conditions, the data were also analyzed with the program SEDPHAT (22). The curves resulting from this latter analysis (Fig. 4) were obtained with the hybrid local continuous distribution model with Tikhonov regularization p = 0.95, allowing the frictional coefficient to float. The curves were normalized by dividing the y-values by the total signal obtained in the c(s) distribution. At least 300 points were used for each curve in the appropriate range of sedimentation coefficients. As the experimental temperature approached the upper limit of temperature that is accessible with the Optima XL-I (40 °C), oil vapor from the vacuum pump collected on the optical elements during data collection, so the optics were cleaned after each experiment.

**Chaperone Activity Assays**—Chaperone activity was measured by monitoring the DTT-induced aggregation of insulin in the absence and presence of Hsp27. Aggregation was initiated by unfolding insulin with the addition of DTT solution (60 μl, 1 mM) to a solution (590 μl) of insulin (0.24 mg/ml) containing varying concentrations of wild-type or variant Hsp27 in sodium phosphate buffer (pH 7.2) that had been incubated for 10 min at the desired temperature. Aggregation was monitored by measuring light scattering at right angles with a Varian Cary Eclipse spectrophotometer equipped with a Peltier device for temperature control. Both the emission and excitation wavelengths were set at 465 nm, and the band pass was 2.5 nm. Kinetics traces reported here are the averages of two measurements. The percentage activity was calculated as 100(\frac{I_{\text{ins}} + I_{\text{ins+Hsp}}}{I_{\text{ins}}}) where I is the intensity of light scattering observed after 30 min.

**RESULTS**

**Self-association of Wild-type Hsp27 and the S15D/S78D/S82D Variant Is Independent of pH and Ionic Strength**—To evaluate the possible role of titratable functional groups in Hsp27 oligomerization, sedimentation velocity experiments were performed as a function of pH from pH 6.5 to 8.4. The results obtained with both the wild-type protein (0.6 mg/ml) and the triple variant (0.6 mg/ml) (Fig. 2, B–D) establish that the self-association of neither wild-type Hsp27 nor the triple variant (as reflected by S_{av}) is dependent on pH between pH 7 and 8.4. Upon further
lowering of the pH to 6.5, the wild-type protein exhibited an increase in $S_{av}$. This behavior is presumably related to the fact that Hsp27 is not soluble at its pl (predicted to be ~6.0) (23).

The self-association of wild-type Hsp27 ($\sim$1 mg/ml) was studied as a function of ionic strength at pH 8.4, and the data were analyzed with the program SEDFIT (21). The variation of the weight average sedimentation coefficients ($S_{av}$) as a function of [NaCl] obtained from this analysis (Fig. 2A) demonstrates that the state of oligomerization does not change significantly upon increasing the ionic strength from 0.05 to 1 M sodium chloride. Similar results were obtained with the S15D/S78D/S82D Hsp27 variant (0.3 mg/ml). This variant, which is a functional mimic of phosphorylated Hsp27 *in vitro* (17), was previously reported to form mainly monomers and dimers in a buffer containing 0.1 M NaCl (18). For this variant, the largest oligomers are observed in the presence of 0.2 M NaCl (Fig. 2C); as for the wild-type protein, a reduction or an increase in the ionic strength changes the $S_{av}$ only slightly.

**Wild-type Hsp27 Exhibits Thermally Induced Self-association**—Sedimentation velocity analyses of wild-type Hsp27 at 10, 30, and 40 °C and protein concentrations ranging from 0.04 to 1.79 mg/ml were compared with corresponding results previously obtained at 20 °C (18). The weight average sedimentation coefficients as a function of protein concentration (Fig. 3A) and the $S_{av}$ versus temperature for a given concentration ($\sim$0.9 mg/ml) are shown (Fig. 3B) as are the $c(s)$ data obtained as a function of temperature (10, 30, and 40 °C) (Fig. 4).

As observed previously at 20 °C, the breadths of the peaks observed in the $c(s)$ distribution at the various temperatures studied in this work are greater than expected for discrete, non-interacting components, and the apparent sedimentation coefficient for each peak varies directly with protein concentration. These results indicate that the boundaries observed are “reaction boundaries” in which the sedimenting species interconvert on a time scale that is at least as great as that of sedimentation. As a consequence, the observed peaks do not correspond to a particular oligomeric assembly but instead reflect some weight average of the sedimenting species. However, by increasing the protein concentration sufficiently, the peak of the $c(s)$ distribution approaches the peak of the largest oligomer. Therefore, the increase in $S_{av}$ with increased protein concentration at all temperatures studied (10–40 °C) is consistent with our previous observations at 20 °C (18) and indicates that at all temperatures, Hsp27 behaves as a self-associating system. Moreover, the fact that the $S_{av}$ seems to reach a plateau indicates that we were able to approach the largest oligomer formed at each temperature.

Surprisingly, however, we also observed that the self-association of Hsp27 exhibits an unusual dependence on temperature such that on increasing the temperature from 10 to 40 °C, even larger oligomers form (Fig. 3, A and B). The $c(s)$ plots (Fig. 4) show that at all temperatures some small oligomers are present in solution, especially at the lowest concentration analyzed. Consequently, we conclude that Hsp27 dissociates into monomers and dimers independent of the temperature at which the experiments are run, whereas the size of the major oligomeric species varies directly with temperature.

The frictional coefficient ($f/f_0$) is a measure of the diffusional boundary spread during sedimentation scaled relative to the $S$ value of the species. The value of this parameter is allowed to float during data analysis with SEDFIT because independent information concerning the average degree of boundary spreading and sedimentation can be extracted from the experimental data (21). The $f/f_0$ value provides an estimate of the extent to which the shape of a protein differs from a compact, unhydrated sphere of the same mass and density (24). A globular, hydrated protein would have a frictional coefficient of 1.2–1.4; a moderately elongated protein (e.g. fibrinectin coiled up in low salt) would have a frictional coefficient of 1.6–1.9, whereas very elongated proteins (e.g. fibrinogen) would have a frictional coefficient of 2–3. Together with the $S$ value, the $f/f_0$ allows estimation of the molecular weight of the sedimenting species (25).

The presence of multiple species in solution complicates the interpretation of the frictional ratio obtained with SEDFIT for a $c(s)$ distribution. In this case, the resulting $f/f_0$ is only an approximation of the true $f/f_0$, because it is calculated as an average of the frictional ratios of all the species present. However, for a sample in which the largest species observed is >90% of the total protein, then the fitted $f/f_0$ is a good approximation of the true frictional ratio of the larger sedimenting species (26). The use of a single average frictional coefficient in the interpretation of the sedimentation data of an oligomerization process has been quite successful, for example, in the study of ligand-linked association of tubulin by Timasheff and co-workers (27). For proteins that interact on the time scale of sedimentation, an additional contribution to boundary spread is the interconversion of species due to the dissociation and re-association of the oligomers (a chemical process). With the exception of repulsive interactions at protein concentrations exceeding those used in the present study, such a chemical process leads to excess boundary spreading, which can result in artificially high apparent diffusion and consequence low frictional ratios at concentrations in the range
of $K_d$ of the interaction (22). It was shown recently in the “constant bath” theory for heterogeneous interactions that the sedimentation/diffusion process of reacting systems may be described as a good approximation of sedimentation and diffusion of non-reacting systems with the apparent sedimentation and diffusion coefficients following a binding isotherm (28, 29). This result suggests that at conditions close to saturation of the largest oligomer, the apparent weight average $f/f_0$ value from $c(s)$ should be close to the true $f/f_0$ value of the largest oligomers. Any error in this approximation would lead to an understimation of the molecular weight and underestimation of the shape asymmetry.

Although the three-dimensional structure of Hsp27 is not known, the few available crystal structures of small heat shock proteins and all results derived from electron microscopy indicate that sHsps are nearly spherical proteins. This characteristic is reflected by the frictional coefficient obtained from analysis of our current data with SEDFIT (1.2–1.4) and molecular weights consistent with the known masses of sHsps. At the highest protein concentrations used in this study, we obtained frictional coefficient ratios ($f/f_0$) of 1.30 (10 °C), 1.37 (30 °C), and 1.22 (40 °C). Assuming these values as the frictional coefficient for the largest Hsp27 oligomers observed at each temperature, we can deduce that self-association results in fairly compact quaternary structures and can estimate the size of the oligomers formed at each temperature. At 10 °C, a broad peak encompasses small $S$ values up to 12 $S$, indicating that the largest sedimenting species is probably no larger than 8–12 mer. At 30 °C, the main peak has an $S$ value that spans 13–15 $S$, indicating that the main sedimenting species is 16–20 mer. At 40 °C, the main component sediments with $S$ of 18–22 $S$, indicating that the main sedimenting species is in the range of 22–30 mer.

Repetition of velocity experiments of wild-type Hsp27 in phosphate buffer (20 mM pH 7.4, 100 mM NaCl, 40 °C) resulted in identical behavior, so this result does not arise from the thermodynamic properties of the buffer (data not shown). The reversibility of this temperature-dependent self-association was demonstrated by the observation that $c(s)$ plots obtained from samples that were heated for 2 h at 42 °C prior to sedimentation velocity analysis at 20 °C exhibited the same degree of self-association as observed for protein maintained at 20 °C (data not shown). This latter result indicates that oligomerization of Hsp27 at elevated temperature is attributable to the formation of well defined, higher order oligomers rather than to nonspecific aggregation. Moreover, incubation at 40 °C for longer periods of time (up to 8 h prior to starting the sedimentation velocity experiments) did not change the upper limit of size of oligomers formed (data not shown). As indicated above, limitations in instrument design prevented use of higher temperatures in these experiments.

The Triple Variant of Hsp27 Responds Minimally to Increased Temperature—To evaluate the influence of temperature and oligomer size on the chaperone activity of Hsp27, the ability of the wild-type and variant proteins to decrease DTT-induced aggregation of insulin was evaluated as a function of temperature. As expected, wild-type Hsp27 exhibits activity that is dependent on protein concentration (Fig. 6A). A ratio of 1.02 (w/w) insulin:Hsp27 is sufficient to inhibit insulin aggregation fully at 20 °C. To evaluate the temperature dependence of this activity, similar experiments were performed at an insulin:Hsp27 ratio of 1.05 (w/w) from 20 to 48 °C (Fig. 6C). Notably, the most acute sensitivity to increasing temperature was observed at temperatures that are relevant to the physiological heat shock response (34–43 °C).

The triple variant was also able to inhibit insulin aggregation in a temperature-dependent manner, but the extent of inhibition is much less than that observed with the wild-type protein (Fig. 6B). At 40 °C and an insulin:Hsp27 ratio of 1.05, the wild-type protein inhibits aggregation by 64%, whereas the triple variant exhibits just 15% inhibition. At the same insulin:Hsp27 ratio but at 20 °C, the triple variant did not inhibit aggregation. These findings are in accord with the observation that larger oligomers have chaperone activity, that the triple variant forms only small amounts of larger oligomers even at high temperature, and that the size of this larger oligomer is temperature dependent for both proteins.

**DISCUSSION**

In view of the complexity of the self-association equilibria exhibited by the wild-type protein, we have used analytical ultracentrifugation as
the method of choice for this study. An essential feature of this approach has been the use of the c(s) analysis provided by the programs SEDFIT and SEDPHAT because the numerical analysis provided by these programs does not require initial guesses or information concerning the system under analysis other than the standard physical parameters (e.g. temperature, buffer viscosity and density, and the protein amino acid sequence). To analyze our data further, we determined the weight average sedimentation coefficients (S_av) from the c(s) plots because this approach has been demonstrated previously to provide a rigorous means of obtaining thermodynamically valid parameters (22). Previous studies of temperature-dependent self-association of heat shock proteins have generally involved incubating the protein of interest at elevated temperature and then evaluating the state of oligomerization by size exclusion chromatography at ambient temperature. In principle, this practice allows sufficient time for at least partial re-equilibration of the system at ambient temperature during the time required for elution of the column. In the case of Hsp27, in fact, we found that the thermally induced formation of larger oligomers is fully reversible, so analysis of the self-association equilibrium of this protein must be performed under temperature-controlled conditions.

Understanding the environmental factors to which the self-association equilibria and chaperone activity of Hsp27 respond is essential to a rigorous understanding of the mechanism by which this protein functions in vivo and to the design of future experiments concerning the participation of various functional domains of the protein to its behavior.

The independence of Hsp27 self-association on pH and ionic strength indicates that oligomerization involves neither titratable groups that titrate in the range studied nor significant general electrostatic stabilization. Results for the S15D/S78D/S82D triple variant established that the behavior of this variant is similarly independent of pH and ionic strength. Thus, although no general electrostatic contribution to self-association seems to be involved in oligomerization of Hsp27, specific electrostatic effects at the residues substituted in the triple variant can effectively impede self-association. On the other hand, the self-association of wild-type Hsp27 exhibits a significant dependence on temperature between 10 and 40 °C that results in formation of larger Hsp27 oligomers with increasing temperature (Figs. 3 and 4). Moreover, this thermally induced oligomerization is fully reversible (data not shown).

Notably, the thermal dependence of Hsp27 oligomerization is reflected in a pronounced thermal dependence of Hsp27 chaperone activity (Fig. 7). Specifically, the chaperone activity of Hsp27 increases slightly between 20 °C (9% inhibition of insulin aggregation) and 30 °C (13.5% inhibition), with much greater increases observed between 34 °C (19% inhibition) and 43 °C (64% inhibition). At higher temperature, little further increase in activity results (67% inhibition at 48 °C (insulin: Hsp27 1:0.05) (Fig. 6C). Although increased chaperone activity of α-crystallin and Hsp22 have previously been reported to increase upon increasing temperature from 20 to 50 °C (30–32), the direct correlation of thermally activated chaperone activity with increased oligomer size has not been reported previously. In view of the fact that Hsp27 expression is thermally induced in vivo in the temperature range where we observe the greatest effects on both oligomerization and chaperone activity, we conclude that these temperature-dependent properties of Hsp27 constitute a previously unrecognized physiological characteristic of this protein.

The corresponding results obtained for the S15D/S78D/S82D triple variant provide mechanistic insight into the role of phosphorylation in regulating Hsp27 behavior. At 40 °C and low protein concentration, this variant forms a mixture of monomers and dimers. At higher protein concentration, a small fraction of the protein forms oligomers that are comparable in size to the largest oligomers observed for the wild-type protein at 40 °C. This behavior is identical to that reported previously at 20 °C (18), so it appears that the inhibition of Hsp27 self-association that results from these electrostatic modifications and from the related electrostatic effects of Hsp27 phosphorylation can inhibit both the thermally induced self-association of the protein and the thermal activation of Hsp27 chaperone activity. Nevertheless, if the phosphorylated variant is present at high local concentration, it is still capable of forming larger oligomers that retain chaperone activity. This observation is consistent with the previous report by Gar-rido and co-workers (33) that this variant exhibits some of the features of the wild-type protein in protecting against cell death in vivo.
Previous studies of other small heat shock proteins have reported dissociation of larger oligomers to form smaller assemblies at elevated temperature (34–37). On the other hand, a few examples of large heat shock proteins that form larger oligomers with increased temperature have been reported. For example, chicken Hsp90 has been reported to be dimeric below 50 °C and to form larger oligomers at higher temperatures (38, 39). The endoplasmic glycoprotein chaperone gp96, which is highly similar to Hsp90 from yeast (40), also forms larger oligomers and exhibits greater peptide binding affinity at temperatures >50 °C (39). To our knowledge, Hsp27 is the only true small heat shock protein and the only ATP-independent heat shock protein currently known to form oligomers of increased size as temperature is increased. Other protein–protein complexes that have been reported to exhibit increased stability at elevated temperature include the self-association of RecA (41), an anti-cytochrome c-cytochrome c complex (42), and cytochrome c-peroxidase complex formation (43).

We conclude that Hsp27 is a small heat shock protein that exhibits a remarkable thermally induced increase in oligomer size that is manifested functionally by a substantial increase in chaperone activity that occurs primarily over the range of temperature that is germane to the heat shock response. Furthermore, this thermal response is largely prevented by phosphorylation. Nevertheless, at sufficiently high protein concentration and temperature, the phosphorylated protein (as indicated by the triple variant) does exhibit some chaperone activity. These results establish a new thermodynamic characteristic of the protein with direct physiological implications, and they provide a mechanistic basis for the ability of the triple variant to protect cells against apoptosis (33).

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