Oligomerization is an essential property of small heat shock proteins (sHSPs) that appears to regulate their chaperone activity. We have examined the role of conserved hydrophobic residues that are postulated to stabilize sHSP oligomers. We identified a mutation of *Synechocystis* Hsp16.6 that impairs function in vivo and in vitro. The V143A mutation is in the C-terminal extension, a region predicted to form an oligomeric interaction with a hydrophobic region that includes the site of a previously characterized mutation, L66A. Both mutants were dimeric, but V143A had a stronger oligomerization defect than L66A. However, V143A protected a model substrate better than L66A. This suggests that although the two regions both play a role in oligomerization, they are not equivalent. Nevertheless, the addition of either dimeric sHSP enhanced the in vitro chaperone activity of wild type Hsp16.6, consistent with models that the sHSP dimers initiate interactions with substrates. Suppressor analysis of V143A identified mutations in the N terminus that restored activity by restabilizing the oligomer. These mutants were allele-specific and unable to suppress L66A, although they suppressed a dimeric C-terminal truncation of Hsp16.6. Conversely, suppressors of L66A were unable to suppress either V143A or the truncation, although they, like suppressors of V143A, stabilize the Hsp16.6 oligomer. We interpret these data as evidence that the mutations V143A and L66A stabilize two different dimeric structures and as further support that sHSP dimers are active species.

Small heat shock proteins (sHSPs) are a family of ATP-independent chaperones that, in vitro, can protect denaturing proteins from irreversible aggregation by forming large, soluble complexes with their substrates (for reviews, see Refs. 1–3). sHSPs do not refold substrates, but sHSP-protected substrates can be refolded by ATP-dependent chaperones such as Hsp70/DnaK (4, 5). Although the role of sHSPs in vivo is not well defined, established in vitro assays for sHSP activity (i.e. protection of model substrates from aggregation and their subsequent refolding by ATP-dependent chaperones) appear to be closely related to sHSP functions in vivo. In *Synechocystis* sp.

Strain PCC 6803 ( *Synechocystis* ), a missense mutation of the single sHSP in this organism, Hsp16.6, causes loss of both protection of a model substrate in vitro and the ability of cells to survive heat treatment (thermotolerance). Both activities are restored by intragenic suppressors of this mutant (6). Mogk et al. (7) demonstrated that the sHSPs in *Escherichia coli* act with both DnaK and ClpB to facilitate the removal of aggregated protein after heat stress, in good agreement with in vitro observations of Hsp70/DnaK-dependent reactivation of sHSP-protected substrates (8, 9). Thus, it appears that sHSPs function to prevent or reduce irreversible protein aggregation during heat stress in vivo.

The mechanism of the chaperone activity of sHSPs is unknown. Current models suggest that changes in sHSP oligomerization are essential. Almost all sHSPs form oligomers (2), and these can be destabilized at high temperatures (10, 11). Disassembly of the oligomer into dimers has been proposed to be a required step in the chaperone process (10). Disassembly could produce an active state of the sHSP that can bind unfolding proteins (10), perhaps by exposure of hydrophobic residues that bind substrates (5, 11, 12). However, the oligomeric state is also essential, as it appears that reassembly of sHSP dimers into a complex with substrate is required for protection of substrates (6, 13–16). Therefore, identification of contacts involved in oligomerization is important for understanding the mechanism of sHSP chaperone activity.

The crystal structures of two sHSPs provide information about regions involved in oligomerization. The structures of *Methanococcus jannaschii* Hsp16.5 (17) and *Triticum aestivum* Hsp16.9 from wheat (18) reveal that despite the difference in the size of their oligomers (24 and 12 subunits, respectively), there is a high degree of similarity between the two sHSPs. Both proteins form oligomers that are composed of dimers, and the conserved ~100 amino acid α-crystallin domains (18) have very similar β-sandwich structures. Oligomerization of *M. jannaschii* Hsp16.5 and *T. aestivum* Hsp16.9 involves a conserved interdimer interface between two hydrophobic regions: a “patch” of hydrophobicity on one surface of the β-sandwich of the α-crystallin domain and the hydrophobic residues in a basic-X/I/V/X/I/V motif of the C-terminal extension (β-strand 10). The conservation of these residues among sHSPs (18) is consistent with essential functions of these regions.

The hypothesis that interactions between the C-terminal extension and the hydrophobic patch are essential to sHSP oligomerization and function is supported by some, but not all, mutagenesis experiments in vitro and has not been tested in vivo. Mutation of either Ile residue to Ala in the basic-X/I/V/X/I/V motif of HspH from *Bradyrhizobium japonicum* destabilized the oligomer and led to loss of protection of citrate synthase from aggregation (15). A truncation of the C terminus that deleted this motif also reduced the oligomerization of
M. jannaschii Hsp16.5 (19) but not of Hsp16.2 from Caenorhabditis elegans (13). Furthermore, while Kim et al. (19) found that the C-terminal truncation of M. jannaschii Hsp16.5 led to formation of a tetramer, it was still able to maintain substrate solubility. The role of the hydrophobic patch is even less well examined. We identified a mutation of a conserved hydrophobic residue in the patch, L66A of Synechocystis Hsp16.6, that severely reduced both oligomeric stability and chaperone activity (6), but this region has not been investigated in other sHSPs. Although on the whole, these studies argue for a role of the hydrophobic C-terminal motif and patch in both oligomerization and chaperone activity, the requirements for either are not clear. Furthermore, these studies have primarily assessed sHSP activity using model substrates in vitro, making it possible that the results do not reflect cellular needs for sHSP activity.

We took advantage of the requirement of Hsp16.6 for thermotolerance in Synechocystis to dissect genetically the role of the C-terminal motif and to compare it with that of the hydrophobic patch. The relationship of in vivo phenotypes to oligomeric stability and chaperone activity was also compared. Point mutants at Val143 (24), the second hydrophobic residue in the basic-X[I/V][X][I/V] motif of Hsp16.6, decreased the viability of heat-stressed cells, providing the first direct evidence for the importance of this motif in vivo. The V143A protein was diclinic and had impaired chaperone activity in vitro. V143A disrupted oligomeric stability more, but chaperone activity less, than L66A, a mutation in the hydrophobic patch, suggesting the two hydrophobic regions do not have equivalent functions. Intragenic suppressors of V143A were then selected in Synechocystis as second-site mutations that restored cellular thermotolerance. These suppressors were located in the N terminus and greatly stabilized the Hsp16.6 oligomer. Contrary to expectations, the suppressors of V143A were not able to suppress L66A. Likewise, suppressors of L66A, which also stabilize the Hsp16.6 oligomer (6), did not suppress V143A. In total, although both V143A and L66A form stable dimers, there are structural and/or functional differences between the two mutants. We suggest that they may stabilize two distinct functional states of Hsp16.6.

**Experimental Procedures**

**Plasmids—**Synechocystis hsp16.6 and its mutants were subcloned into the HpaI and ApaI sites of the plasmid pNaive (6) containing a spectrin mincin resistance marker for transformation into Synechocystis. For expression in *E. coli*, the genes were inserted into the same sites in pUC20/Hpa, a modified version of pUC20 (20) described previously (6).

Site-directed mutagenesis of Val143 was performed by PCR with a degenerate oligonucleotide that could mutate hsp16.6 codon 143 to code for Gly, Ala, Ser, Thr, and Arg, using pUC/Hpa hsp16.6 as a template. A pool of the HpaI/ApaI fragments of the PCR product was then cloned into pNaive as above.

**Synechocystis Strains and Growth Conditions—**Synechocystis sp. strain PCC 6803 was grown at 30 °C on BG-11 medium (21), supplemented with 5 mM glucose, as described previously (6). The strain HK-1/ΔClpB1, which carries a deletion of both hsp16.6 and clpB1, was used as the parental strain in all Synechocystis transformations. Cells were transformed as described by Williams (22), with pNaive (to create the Hsp16.6 deletion strain), pNaive16 (for the wild type [WT] Hsp16.6-expressing strain), or mutant variants and selected for increasing spectrin mincin resistance at concentrations up to 250 μg/ml spectrin mincin dihydrochlordiaz.

**Synechocystis Thermotolerance—**Assays were performed as previously described (6). Briefly, cells growing at log phase were serially diluted five times at 1:10, and 5 μl of each dilution was spotted onto plates containing 20 ml of BG-11/agar with 140 mM MgSO₄ and 5 mM glucose. Cells were heated at 44 °C for up to 10 h and then transferred to a lit 30 °C growth chamber until colonies became visible. Colony numbers were compared with unstrained samples to calculate the viability of each sample.

Hsp16.6 Accumulation in Synechocystis—Log phase cells were incubated at 2 h at 42 °C. Cells were pelleted and lysed in SDS sample buffer (30 mM Tris, pH 6.8, 5% glycerol, 2% SDS, 0.01% bromophenol blue, 2.5% β-mercaptoethanol). Protein concentration was determined by Coomassie Blue binding (23). Total cellular protein (0.5 μg) was loaded onto an 15% SDS-PAGE gel, which was immunoblotted with anti-Hsp16.6 antiserum (6).

**Protein Purification—**Hsp16.6 and mutant variants were purified from the *E. coli* strain BL21 (Strategene). Purification was performed as described previously (6), except that an additional purification step was added. Protein eluted from a DEAE column was diluted, and salt was added to bring it to 4 mM NaCl and 1.5 mM urea. Protein was loaded onto a 5-ml HiTrap Butyl column (Amersham Biosciences) equilibrated in 50 mM NaPO₄, pH 7.3, 2 mM NaCl, 1.5 mM urea, and 1 mM dithiothreitol and eluted with a 4 to 0 mM NaCl gradient. Protein was stored in 20 mM NaPO₄, pH 7.3, 20 mM NaCl, and 1 mM dithiothreitol.

The concentration of WT Hsp16.6 was determined using an extinction coefficient ε₅₉₀ = 5980 M⁻¹ cm⁻¹ (6). Mutant proteins that had no contaminants detectable by Coomassie Blue stain were measured similarly, except that the extinction coefficient ε₅₉₀ = 7450 M⁻¹ cm⁻¹ (calculated as described by Pace et al. (24)) was used for proteins with S2Y or N7Y mutations. Proteins with detectable contaminants (but typically at least 95% pure) were assayed by the method of Bradford (25), using WT Hsp16.6 as the standard.

**Size Exclusion Chromatography (SEC)—**SEC was performed by high pressure liquid chromatography. 100 μl of 24 μM sHSP samples were injected onto a Bio-Sil SEC 400-5 column (Bio-Rad) equilibrated with 20 mM NaPO₄, pH 7.3, 20 mM NaCl or with 200 mM NaPO₄, pH 7.3, 200 mM NaCl, when looking at sHSP-substrate complexes. The column flow rate was 1 ml/min. Buffer and column were maintained at room temperature. Samples were heated as described and centrifuged 15 min, 16,000 × g before being injected onto column. Thyroglobulin (670 kDa), β-amylase (200 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and myoglobin (17 kDa) were used as molecular weight standards. Proteins were detected by absorbance at 220 nm.

**Cross-linking of Hsp16.6—**Glutaraldehyde cross-linking was performed based on the method of Craig (26). 48 or 192 μM Hsp16.6 in 20 mM NaPO₄, pH 7.3, 20 mM NaCl was heated at 42 °C for 7.5 min and cooled briefly on ice. A 250-fold molar excess of glutaraldehyde was added, and reactions were incubated at room temperature for 1 h. Reactions were quenched by the addition of a 10-fold molar excess of glycine over glutaraldehyde. 30 μg of sHSP were run on 5–15% SDS-PAGE gels and Coomassie Blue-stained.

**Luciferase Protection Assays—**Firefly luciferase (Luc) reactivation was performed as described previously (6). Mixtures of Hsp16.6 and luc at concentrations indicated were prepared in 25 mM HEPES, pH 7.5, 15 mM KCl, 5 mM MgCl₂, and 2 mM dithiothreitol (D buffer) and were heated at 42 °C for 7.5 min. In refolding experiments, samples were diluted to a final sHSP concentration of 30 μM in 80% rabbit reticulocyte lysate (BRL) buffer with 2 mM ATP and allowed to refold at 31 °C. Luciferase activity was measured at time points from 0 to 2 h. To determine aggregation-protection, samples were centrifuged (15 min, 16,000 × g at 4 °C) directly after heating, and soluble and pelleted fractions were run on 15% SDS-PAGE.

**Hsp16.6 Solubility—**Resistance of sHSPs to aggregation at high temperatures was tested by heating 24 μM sHSP in D buffer at 55 °C for 24 h. Samples were then centrifuged for 15 min at 16,000 × g at 4 °C, and the amount of protein in the soluble fractions was compared with unheated samples after SDS-PAGE and Coomassie Blue staining.

**Isolation of Suppressors—**Random mutagenesis of the hsp16.6 V143A gene was performed with manganese-based, degenerate PCR, and isolated genes were transformed previously (6). Mutant genes were cloned into the plasmid pNaive, and pools of plasmids were transformed into the Synechocystis strain HK-1/ΔClpB1. Plasmid integration was selected for with increasing spectrin mincin, up to 250 μg/ml, before replicating colonies to plates containing 140 mM MgSO₄. Plates were heated at 44 °C for 10 h and allowed to recover for up to 10 days. Only clones that survived to a significant degree, creating large patches of growth, were selected as putative suppressors. Genomic DNA was isolated from these cell lines and used to amplify the hsp16.6 gene for sequencing. Mutants were recloned into pNaive and transformed into HK-1/ΔClpB1 cells, in order to verify heat resistance using the quantitative assay described above.

**RESULTS**

**Mutations at Val143 Are Deleterious to in Vivo Function of Hsp16.6—**As described above, sHSP interactions between the
Mutants of Synechocystis Hsp16.6 That Affect Oligomerization

hydrophobic patch in the α-crystallin domain and conserved hydrophobic amino acids in the C-terminal extension are postulated to be crucial for oligomerization and chaperone activity. In order to test for a requirement for this interaction in cellular thermotolerance, we examined the effect of mutagenesis of Val143 in the Synechocystis sHSP, Hsp16.6. Val143 is the second of the two hydrophobic residues in the conserved motif, basic-XI/XII/XIII, in the C-terminal extension. It was also of interest to compare mutations of Val143 to those of L66A in the hydrophobic patch, which we characterized previously (6). Based on the M. jannaschii Hsp16.5 structure, the residues Leu66 and Val143 in Hsp16.6 may be in close proximity in this oligomeric interface (17). Therefore, comparison of these mutants would test the hypothesis that these two hydrophobic regions share a single function, namely oligomerization, both in vivo and in vitro.

In all experiments, we used a strain of Synechocystis with a deletion of clpB1 due to its enhanced sensitivity to Hsp16.6 activity (6). Because the parental strain is also hsp16.6, transformation with an hsp16.6 mutant creates a strain that expresses only the mutant sHSP. Synechocystis colonies transformed with hsp16.6 containing random mutations at Val143 were screened for loss of thermotolerance. Colonies that did not survive a heat treatment of 8 h at 44 °C for 2 h were isolated from replicate, unstressed plates and used to recover the hsp16.6 gene for sequencing. V143A, V143G, V143S, and V143T were identified as loss-of-function mutants. All four mutants have similar phenotypes in vivo, with viabilities of 0.5–2% after 8 h at 44 °C compared with −30% for the WT Hsp16.6 strain (data not shown). None of these mutations of Val143 had an effect on Hsp16.6 accumulation in Synechocystis at 42 °C (Fig. 1A). Therefore, the loss of cell viability during heat stress is due to changes in sHSP function, not abundance. These data are consistent with an essential function of the C-terminal motif in Hsp16.6.

These mutations all reduce the hydrophobicity at residue 143, suggesting that this is the critical change. It is possible that the β-branched nature of Val (and Ile, which is often found in this position of the motif) is an essential feature at this position. However, since Thr is also β-branched and cannot substitute for Val, a β-branched residue is clearly not sufficient for sHSP function. Due to the similarity of phenotypes of all four mutants, only the most conservative mutation, V143A, was analyzed further. As shown in Fig. 1B, after 10 h at 44 °C, the Hsp16.6 V143A strain is ∼100-fold less viable than +Hsp16.6, a strain expressing WT Hsp16.6, and ∼100-fold more viable than the ΔHsp16.6 strain. The V143A strain is also more thermotolerant than the one that expresses the L66A mutant, which may reflect differences in the activities of the mutants, but could also be the result of the reduced accumulation of L66A (Fig. 1A).

V143A Destabilizes the Hsp16.6 Oligomer—The V143A protein was compared with WT Hsp16.6 and the L66A mutant by SEC, in order to relate in vivo activity to oligomeric stability. SEC of 24 μM sHSP performed at room temperature showed that the majority of the V143A protein eluted at a time consistent with a dimer, under conditions where WT Hsp16.6 was oligomeric (Fig. 2A). L66A was more oligomeric than

![Figure 1](image1.png)

**Fig. 1.** Mutations of Hsp16.6 Val143 cause thermosensitivity in Synechocystis without affecting sHSP accumulation. A, accumulation of Hsp16.6 in Synechocystis was determined by Western blot of lysates of cells heated at 42 °C for 2 h, as described under “Experimental Procedures.” B, average survival of the Synechocystis strains +Hsp16.6 (circles), ΔHsp16.6 (triangles), and strains expressing the V143A (diamonds) or L66A (squares) mutants on plates, containing 140 mM MgSO4, incubated at 44 °C for up to 10 h. All four strains are ΔclpB1. The error bars show the S.E. between plates from three independent experiments and a total of nine samples per time point.

![Figure 2](image2.png)

**Fig. 2.** V143A severely disrupts Hsp16.6 oligomerization. A and B, SEC elution profiles of 24 μM Hsp16.6 WT (closed circles), V143A (diamonds), and L66A (squares) at room temperature. Samples were kept at 4 °C (A) or heated at 42 °C for 7.5 min and then cooled at 4 °C for 20 min (B) before loading on the column. The column was equilibrated with 20 mM NaPO4, pH 7.3, 20 mM NaCl. Elution times of the molecular weight standards described under “Experimental Procedures” are shown with open circles. C, Coomassie Blue-stained gradient (5–15%) SDS-polyacrylamide gel of glutaraldehyde-cross-linked Hsp16.6. 48 or 192 μM Hsp16.6 was heated and cooled as in B and then exposed to glutaraldehyde for 1 h at room temperature, as described under “Experimental Procedures.” 30 μg of protein was loaded per lane.
V143A and eluted as a mixture of oligomer and dimer. Both mutant proteins were destabilized further by heating; after heating and cooling (42 °C for 7.5 min, followed by 20 min at 4 °C prior to injection onto the column), V143A eluted entirely as a small species (Fig. 2B). Although L66A also appeared to be fully dimeric after heat treatment, it eluted more slowly than V143A. The difference in their elution times may be due to an interaction of L66A with the column matrix and/or structural differences.

In order to determine whether the small species of V143A and L66A both represent dimers, glutaraldehyde cross-linking was performed on 48 μM sHSP samples that had been heated and cooled as above. The resulting species were analyzed by SDS-PAGE (Fig. 2C). After a 1-h exposure to glutaraldehyde, the oligomeric WT Hsp16.6 was cross-linked to form a variety of species, and a significant fraction was larger than tetramers. In contrast, the majority of both L66A and V143A were cross-linked as dimers, although small fractions of both proteins cross-linked as trimers, tetramers, and larger species. This indicates that whereas both mutant proteins were significantly dimeric after heat treatment, consistent with the SEC results, glutaraldehyde could trap both proteins in larger structures during the 1-h incubation. There is a reproducible difference between L66A and V143A in the appearance of the dimer bands. The dimer of L66A consistently ran as a diffuse band, relative to the more compact band in the V143A sample.

**Dimeric sHSPs Have Different Chaperone Activities in Vitro**—V143A provides better cellular thermostolerance than L66A, yet it appears to be more defective than L66A at oligomerization. In order to understand these distinctions better, we compared the chaperone activities of these two mutants in vitro using firefly luc as a heat-sensitive model substrate. When heated in the presence of an sHSP, luc can subsequently be refolded by ATP-dependent chaperones, such as those found in reticulocyte lysate, to a far greater extent than when luc is heated alone (5). We compared the protection provided by the mutant sHSPs over a range of concentrations (Fig. 3A). The protection of 1 μM luc provided by 24 μM L66A or V143A was very low (6.3 ± 1.3 and 6.0 ± 0.3%, respectively), similar to the reactivation of luc heated in the presence of an equal weight of bovine serum albumin (4.3 ± 1.6%). In contrast, 50 ± 7% of the WT-protected luc was refoldable.

Although at 24 μM V143A was unable to protect luc, its activity was greatly enhanced by raising the concentration. We tested concentrations as high as 240 μM sHSP, preserving a constant molar ratio of 24 sHSP:1 luc. The chaperone activity of V143A was low at ≤48 μM but improved significantly at higher concentrations. At 144 μM and higher, V143A was able to protect luc so that at least 65% could be refolded, which is even slightly better than the 50% refolding from WT. Thus, the protection defect of V143A is concentration-dependent. In contrast, the effect of raising the concentration of L66A was small. While 24 μM L66A allowed 6.1% of the 1 μM luc to refold, 240 μM L66A protected only 9.0 ± 0.9% of the 10 μM luc. We have never observed the activity of L66A to approach that of WT at this 24:1 ratio, although higher concentrations of L66A can protect 1 μM luc nearly as well as WT (6).

As observed previously for L66A (6), V143A allowed luc to refold significantly faster than WT-protected luc, with half of the refolding completed in less than 10 min versus nearly 30 min with WT (Fig. 3B). The experiment shown was performed with samples where 240 μM sHSP was heated with 10 μM luc. At low concentrations of V143A, where it was a poor chaperone, the rates of refolding were also rapid (data not shown). Thus, both dimeric sHSPs allow luc to refold rapidly.

The concentration dependence of the chaperone activity of V143A suggested that it might be oligomeric at high concentrations. We performed glutaraldehyde cross-linking of heat-treated sHSP at 192 μM (Fig. 2C), a concentration at which V143A protects luc somewhat better than WT does and much better than L66A does (Fig. 3A). Significant fractions of V143A and L66A cross-linked as large multimers (>200 kDa), but both remained more dimeric than WT. This indicates that V143A is able to function well as a chaperone under conditions where it is less oligomeric than WT Hsp16.6.

In this high concentration cross-linking experiment, there was a concern that nonspecific cross-linking could occur. We used a mutant that is severely impaired in oligomerization to control for this. K142stop, which is missing the last five amino acids of the C-terminal extension, ran exclusively as a dimer by SEC, even before heat treatment (data not shown). In Fig. 2C, K142stop cross-linked predominantly as a dimer at both 48 and 192 μM, suggesting that the high molecular weight species observed with 192 μM L66A and V143A are indeed the result of assembly of these proteins into large structures.

The cross-linking and SEC data demonstrate that after heat treatment V143A and L66A are similar but not identical in oligomerization and do not explain the advantage that V143A has over L66A at protecting luc at high concentrations or during heat stress in vivo. Therefore, we considered that there might be functional differences between the two mutants that are distinct from their differences in oligomeric stability. The accumulation of L66A in Synechocystis is significantly lower than that of WT and V143A (Fig. 1A). We considered that L66A might be less stable than the other sHSPs, which could account for its low activity both in vivo and in vitro. We looked for protein instability by heating 24 μM samples of all three proteins at 55 °C. Even after 24 h at 55 °C, L66A and V143A remained soluble (data not shown), and we have never observed precipitation of either mutant after purification. Furthermore, SEC analysis showed that L66A and V143A remained dimeric after the 55 °C treatment (data not shown). Therefore, it appears unlikely that the poor activity of L66A is due to dramatic differences in stability.
V143A Forms Complexes with luc—It is well established that heating sHSPs with heat-labile substrates leads to the formation of large, soluble sHSP-substrate complexes (4, 27). The L66A mutant does not form complexes with luc that can be detected by SEC, even under conditions where it protects luc from aggregation (6). Therefore, we were interested to determine whether V143A formed stable complexes under conditions where it protected luc as well as WT (as in Fig. 3B). 240 μM sHSP was heated with 10 μM luc and analyzed by SEC at room temperature. The high protein concentration made it necessary to dilute the samples 10-fold prior to injection onto the column. Both WT and V143A formed detectable large molecular weight complexes with luc (Fig. 4). However, relative to the WT-luc sample, less of the V143A-luc sample eluted from the column, and a significantly smaller amount of complexes was detected, as if the V143A-protected luc had bound to the column. The differences observed between WT and V143A could be due to dissociation of the V143A-luc complexes as a result of the dilution step prior to injection onto the column. To test this, we examined complexes by SEC 24 h after the 10-fold dilution and found no differences in the elution of these and the samples injected immediately after dilution (data not shown). This demonstrates that aggregation of V143A-protected luc is not occurring in solution at low concentration and suggests instead that the complexes formed by V143A are less efficient than WT at preventing irreversible interactions of heat-denatured luc with the column matrix.

Dimeric sHSPs Can Augment the Activity of WT Hsp16.6 in Vitro—The proposal that the dimeric form of the sHSP interacts with substrate (10) suggests that mutants that destabilize the oligomer should still interact with substrate, even if they are impaired at forming stable sHSP-substrate complexes and so ultimately fail to protect substrate. We reasoned that such an interaction with substrate might be detected by an enhancement of the protective activity of WT. To best observe such an enhancement, we used conditions (1.5 μM luc with 12 μM sHSP) under which WT protected less than half of the added luc from becoming insoluble (Fig. 5A, lane 1), and L66A and V143A had essentially no protective activity (lanes 7 and 8). As expected, more luc was soluble when heated in the presence of 12 μM WT plus 12 μM V143A (lane 5) than when it was heated with 12 μM WT alone. Whereas V143A provided better enhancement of solubility than L66A, L66A was also able to assist WT in protecting luc (lane 4), substantiating that both mutants interact productively with substrate. In contrast, the protection of luc that was heated in the presence of both V143A and L66A, but without WT (lane 6), was very poor, demonstrating that the activity of V143A does not complement that of L66A. Nevertheless, 24 μM WT (lane 9) was better at protection than the mixed samples.

We also examined the ability of this protected luc to be reactivated. When protected by 12 μM WT, only 23 ± 3% of the 1.5 μM luc could be reactivated by reticulocyte lysate. When the heating reaction with WT was supplemented with 12 μM V143A or L66A, luc reactivation consistently increased, with a mean of 35 ± 8 and 30 ± 7%, respectively. Since luc heated with a 12 μM concentration of either mutant protein alone refolded to only 2%, these increases are greater than the reactivation predicted if the sHSPs worked independently. Although the extent of refolding varied between experiments, there was a reproducible increase that occurred by adding either L66A or V143A to WT. Therefore, we conclude that the activities of both dimeric sHSPs, while not sufficient for efficient protection on their own, can complement the activity of WT at low concentrations. This sort of complementation has not been observed previously between sHSPs and supports the model that dimers initiate interactions with substrates.
In Fig. 5B, sHSP-luc complexes created as described above were examined by SEC at room temperature. The amount of complex observed from the luc and WT-only sample (solid gray line) was less than that observed when luc was heated in the presence of WT and V143A (solid black line), consistent with observed differences in luc solubility (Fig. 5A). However, despite the advantage that adding V143A conferred, the majority of V143A still eluted as a dimer (compare the 10.6-min peaks of the solid and dotted black traces), indicating that V143A either was not incorporated into the complexes or dissociated from the complexes after they formed. These data suggest that the association of V143A with luc is weaker than that of WT, allowing WT to replace V143A in mixed populations.

In Vivo Defect of V143A Is Suppressed by Mutations at the N Terminus of Hsp16.6—to determine whether the loss of function of V143A in vivo is due to its dimeric state, we next isolated intragenic suppressors of V143A. Suppressors should restore the essential in vivo activity of Hsp16.6 that is missing in the mutant. If V143A, like L66A (6), is suppressed by mutations that increase oligomeric stability, this would be further evidence that the ability to oligomerize is critical for sHSP activity.

We performed random mutagenesis on the hsp16.6 V143A gene and transformed the resulting pools of mutants into Synechocystis. Colonies were heated at 44 °C for 10 h, and survivors were selected. From greater than 1000 colonies tested, only three different suppressors were isolated in this selection: S2Y, N7Y, and a double mutation P29L/N114D. The N7Y mutation was isolated twice, from independent pools.

Suppression of the stress sensitivity of V143A and accumulation of the suppressor mutants in vivo are shown in Fig. 6. Synechocystis cells expressing V143A/S2Y or V143A/N7Y were at least 30-fold more viable than cells carrying V143A alone and accumulated similar amounts of sHSP. P29L/N114D suppressed V143A by less than 10-fold, so this weak suppressor was not characterized further.

V143A Is Suppressed by Mutations That Stabilize the Hsp16.6 Oligomer—in order to determine whether the suppressors of V143A stabilize the Hsp16.6 oligomer, 24 μM purified V143A/S2Y and V143A/N7Y were examined by SEC at room temperature. The unheated double mutants eluted predominantly as oligomers but had larger proportions of small species than WT (Fig. 7, solid lines). After heating at 42 °C for 7.5 min, the majority of V143A/S2Y eluted after 8.6 min and was clearly smaller than WT, which eluted at 8.4 min (Fig. 7, dashed lines). V143A/N7Y was even more disrupted by heating, and more than 70% of it eluted at 9.8 min, consistent with a dimer. Thus, V143A/S2Y and V143A/N7Y are each more oligomeric than V143A but are less oligomeric than WT Hsp16.6, both before and after heat treatments. Similar behavior has been observed for L66A suppressed with either D80V or V108L (6), suggesting a common mechanism of suppression in which the suppressors readjust the equilibrium between oligomers and dimers but without fully restoring WT stability.

We also examined proteins containing the S2Y or N7Y mutation alone and found that each formed oligomers that were much more stable than WT Hsp16.6. The WT oligomer was slightly perturbed by a 42 °C, 7.5-min heat treatment, as shown by the slight change in the elution profile (Fig. 7). Both S2Y and N7Y were less affected by this treatment. Furthermore, SEC at 44 °C demonstrated that both mutants retained significant oligomerization under conditions where the WT oligomer was unstable (data not shown). Therefore, the S2Y and N7Y mutations increase the oligomeric stability of Hsp16.6, both in the presence and in the absence of the V143A mutation.

The chaperone activity of V143A was also restored by the S2Y and N7Y mutations. Reactivation of 1 μM luc protected by 24 μM sHSP increased from 6% with V143A to 52 ± 10 and 68 ± 5% with V143A/S2Y and V143A/N7Y, respectively (Table I and data not shown). These double mutants protected luc for reactivation at least as well as WT Hsp16.6 in parallel experiments (54 ± 2%), indicating that the in vitro chaperone activity of V143A was fully restored by either suppressor. Thus, the sec-

![Fig. 6](image-url) The V143A mutation is suppressed by S2Y or N7Y. A. viabilities of Synechocystis strains after heating for 10 h at 44 °C, as in Fig. 1. Data are the average of three experiments, with four plates per experiment. B. Viabilities of Synechocystis strains after heating for 2 h.

![Fig. 7](image-url) Oligomerization defect of V143A is suppressed by S2Y or N7Y. SEC was performed at room temperature as in Fig. 2. 100 μl of 24 μM Hsp16.6, either unheated (solid lines) or 20 min after a 42 °C, 7.5-min heat treatment (dashed lines) was injected. Elution times of molecular weight standards are shown at the top.
Mutants of Synechocystis Hsp16.6 That Affect Oligomerization

We have not saturated the selections for suppressors of either V143A or L66A, so we directly tested whether the suppressors of L66A can suppress the V143A mutant and vice versa. We created double mutants pairing V143A with T76I, D80V, or V108L, all suppressors of L66A (6), and L66A with S2Y or N7Y. These double mutants were transformed into Synechocystis, and their abilities to confer thermostolerance were tested. As shown in Fig. 8, the suppression by the two groups of secondary mutations was strikingly different. Whereas S2Y and N7Y suppressed V143A by at least 40-fold, these mutants provided only a small increase in viability (less than 10-fold) in the presence of the L66A mutation. Furthermore, T76I, D80V, and V108L provided almost no advantage in the presence of V143A, compared with their 70-fold or greater suppression of L66A. Therefore, the suppressors of L66A and V143A are allele-specific in vivo.

**Allele-specific suppression of dimeric sHSPs in vivo and in vitro**

| Synechocystis thermotolerancea | SEC elution timeb | Luciferase reactivationc |
|-------------------------------|------------------|-------------------------|
| Wild type                     | +++              | 8.4 (O)                 |
| V143A                         | ++               | 9.9 (D)                 |
| V143A/D80V                    | +                | 8.6 (I)                 |
| K142stop                      | ++               | 9.9 (D)                 |
| K142stop/S2Y                  | +++              | 9.0 (I)                 |
| L66A                          | −                | 10.1 (D)                |
| L66A/S2Y                      | +                | 9.8 (D)                 |
| L66A/D80V                     | ++               | 8.9 (I)                 |

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*a* Relative thermostolerance of Synechocystis strains at 44 °C, where wild type = +++.

*b* Elution times of 24 μm sHSP run on an SEC column at room temperature 20 min after 42 °C, 7.5-min heat treatment. Estimated oligomeric structures are given in parenthesis. O, oligomer; D, dimer; I, intermediate species.

*c* Luciferase reactivation by reticulocyte lysate, after heating 1 μl luc with 24 μm sHSP. Means and S.D. values from three experiments are shown.

Eluted peak was very broad and probably due to more than one species of multimer. Elution times correspond to absorption maxima.

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**Fig. 8. Predicted locations of mutated residues of Hsp16.6.** The structure of a dimer of *F. tularensis* Hsp16.9 (11) is shown with monomers in gray and purple, and the C-terminal extension from an adjacent dimer (C') is shown in light yellow. Space-filled a-carbons of homologous residues of Hsp16.6 residues Val143 (blue), Leu66 (red), suppressors of V143A (yellow), and suppressors of L66A (green) are labeled with the name of Hsp16.6 residues. This figure was made using Swiss-Pdb-Viewer (38).

End-site mutations that were selected for their ability to restore V143A activity in vivo also restore protection to a model substrate in vitro. These data support the hypothesis that the ability to form oligomers is somehow essential for sHSP activity both in vivo and in vitro.

**Suppressors of V143A and L66A Are Not Interchangeable—** The data for the suppressors of V143A presented thus far are comparable with what we observed previously for suppressors of L66A (6). Since both sets of suppressors restore oligomeric stability to Hsp16.6, the fact that none of the suppressors of V143A were the mutations identified as suppressors of L66A was unexpected. The suppressors of V143A are located at the N terminus (Fig. 8). The suppressors of L66A are not in the N terminus, with the exception of P8L, which was isolated as part of a triplet mutant and suppresses L66A relatively weakly in vivo on its own. The majority of the suppressor mutations of L66A, including T76I and D80V, are instead located on a face of the dimer (Fig. 8), which we interpreted as evidence for its role in oligomerization (6).
ined by SEC at room temperature. The double mutants V143A/D80V and K142stop/D80V eluted at 9.9 min, nearly identically to the V143A and K142stop proteins. L66A/S2Y eluted faster than L66A, with an elution time of 9.8 versus 10.1 min, but this result is also consistent with a dimer. In contrast, K142stop/S2Y formed species of intermediate sizes between oligomer and dimer, similar to what has been observed for the suppressed proteins V143A/S2Y and L66A/D80V (Table I).

The chaperone activities of the mutant proteins were tested by heating 1 μM luc with 24 μM sHSP. While the suppressed double mutants, V143A/S2Y, K142stop/S2Y, and L66A/D80V, were able to protect luc at least as well as WT, allowing more than 50% of the luc to refold, the chaperone activities of V143A/D80V, K142stop/D80V, and L66A/S2Y were little better than those of V143A, K142stop, and L66A (Table I). All of these latter double mutants protected luc so poorly that no more than 10% could be refolded. However, at higher ratios of sHSP:luc, we did observe improved activity of V143A/D80V relative to V143A (data not shown). This suggests that there is partial suppression of V143A by D80V, although it was clearly not sufficient for protection under our standard conditions in vitro or in vivo. Overall, mutations that are unable to suppress a particular mutant in vivo also did not restore oligomeric stability or chaperone activity.

DISCUSSION

sHSPs form oligomers that are in equilibrium with smaller species, and both states appear to be essential to the activity of sHSPs to prevent aggregation of denaturing proteins (10, 11, 13, 28). This dynamic nature complicates the study of this family of chaperones. Although structural data for two sHSPs have indicated regions of these proteins that make oligomeric contacts (11, 17), the requirement of these contacts for sHSP activity has not been thoroughly tested. Furthermore, structural differences between the crystallized homo-oligomers and sHSP-substrate complexes may result in different interactions between sHSP molecules in the two assemblies, leaving potentially essential interactions between sHSP subunits unknown.

We have approached these problems by identifying mutations of an sHSP that stabilize the normally ephemeral dimeric state. The interaction between hydrophobic residues in the C-terminal extension and the patch on the surface of the α-crystallin domain was chosen for study because it appears to be a conserved interaction that stabilizes the oligomer (11), but its role in chaperone activity is not well characterized. Furthermore, selections for mutations that restore oligomerization and chaperone activity to mutants that form stable dimers have identified additional residues that are also involved in oligomerization in the sHSP Synechocystis Hsp16.6.

Comparison of Mutations That Stabilize the sHSP Dimer—We have shown that a mutation of Val143 causes oligomeric instability and impairs the chaperone activity of Hsp16.6. This is the first demonstration of a requirement for the C-terminal basic-X/IV/XI/IV motif in vivo. The V143A defect in vitro is also in agreement with the experiments of Studer and Narberhaus with an sHSP from B. japonicum (15). Together these results provide evidence that biochemical as-

Fig. 9. Suppressors of V143A are not interchangeable with those of L66A. A, survival of 10-fold serially diluted cells grown at 30 °C only or with a 44 °C stress for 8 or 10 h. B, suppression of L66A (dark bars) and V143A (light bars) by selected mutants was calculated by dividing the viability of Synechocystis strains expressing double mutants of Hsp16.6 by the viability of L66A or V143A strains after heat stress as in A. The survival of +Hsp16.6 relative to L66A and V143A is shown for comparison. Data are averages from three independent experiments.
assessments of oligomer stability and chaperone activity reflect properties that are essential for sHSP function in cells.

We compared the effects of V143A, in the C-terminal extension, with those of L66A, which lies in the hydrophobic patch and also impairs oligomerization and chaperone activity of Hsp16.6 (6). The two mutants were not equally severe; L66A was more deleterious to sHSP function both in vivo and in vitro (Figs. 1B and 1C). However, the oligomeric defect of L66A was actually weaker than that of V143A (Fig. 2A), although both proteins were almost fully dimeric after heat treatment (Fig. 2B). Therefore, while the data demonstrate that each of the two conserved hydrophobic regions have a role in oligomerization, the differences in the activity of the mutants are not explained solely by differences in oligomeric stability.

The hydrophobic patch may have a second function that is not shared by the C-terminal extension, potentially substrate binding, which has been proposed for this region previously (5, 29). However, we have not been able to compare the effects of the mutants on substrate binding, because no direct assays for binding of aggregating protein have been developed for sHSPs. Suppression of both L66A and V143A correlated with increased oligomeric stability (Table I), supporting a role for each region in oligomerization, which is clearly essential for sHSP activity. However, if oligomeric contacts and substrate binding sites overlap, as has been suggested previously (11), suppressors of L66A may enhance substrate binding in addition to increasing oligomeric stability.

Despite their poor chaperone activity at low concentrations, both V143A and L66A can work with WT Hsp16.6 to increase substrate protection. Under conditions where sHSP was limiting, 12 μM WT plus 12 μM V143A protected luc better than 12 μM WT alone (Fig. 5A). L66A was also able to assist WT Hsp16.6, but to a lesser extent. We suggest that the reason for this enhancement is that, under our experimental conditions, the stability of the WT oligomer results in insufficient dimer to bind luc before it aggregates. This deficiency would be masked in the presence of either dimeric sHSP, provided that the mutant can interact with luc. These data are consistent with the model that sHSP dimers interact with unfolding substrate (10) and provide evidence that both mutants retain aspects of chaperone function. Therefore, these mutants may prove useful for studying the initial interactions of sHSPs with substrate in the absence of complex assembly.

Although luc reactivation was comparable when it was protected by high concentrations of WT or V143A (Fig. 3A), the V143A-protected luc appeared to be vulnerable to binding the column irreversibly during SEC (Fig. 4). Furthermore, the sHSP-luc complexes formed in the presence of a mixture of WT and V143A appeared to have little incorporated V143A (Fig. 5B), as if the initial interaction of V143A with luc was transient despite its protective activity. sHSP molecules can dissociate from complexes (30, 31), and dissociation may be increased by the V143A mutation. Luc that was protected by either V143A or L66A, which has not been observed to form stable complexes with luc (6), refolded in reticulocyte lysate much faster than luc protected by WT (Fig. 3B) (6). Thus, mutations that reduce oligomeric stability appear to destabilize sHSP-substrate complexes and in doing so increase the accessibility of the substrate to either the column or to other chaperones, allowing rapid reactivation.

Suppressor Analysis of Dimeric sHSPs—We performed a selection in Synechocystis for intragenic suppressors of V143A. These suppressors restored oligomerization as well as chaperone activity to V143A (Figs. 6 and 7). The suppressors of V143A are mutations of the N terminus, S2Y and N7Y. The N termini of sHSPs are known to play a role in oligomerization. The crystal structure of T. aestivum Hsp16.9 resolved six N termini of 12, which lie in the center of the oligomer and are intertwined between dimers, suggesting that they contribute to oligomeric stability (11). Furthermore, both phosphorylation of the N terminus of mammalian Hsp27 (14, 32) and a mutation of a hydrophobic residue (Phe to Ser) in the N terminus of bovine αB-crystallin (33) decrease oligomeric stability of these sHSPs. S2Y and N7Y are the first mutations of the N terminus to significantly stabilize an sHSP oligomer. The ability of the suppressors to restore chaperone activity implies that the N terminus also contributes to the formation of stable sHSP-substrate complexes.

Surprisingly, the suppressors of V143A are located at different residues from the suppressors of L66A (Fig. 8), although L66A, like V143A, is suppressed by mutations that restore oligomerization (6). Suppressors of V143A do not suppress L66A either in vivo or in vitro, and vice versa (Fig. 9, Table I). Furthermore, analysis of K142stop, a dimeric C-terminal truncation of Hsp16.6, showed that this mutant has the same requirements for suppression as V143A (Fig. 10, Table I). The allele-specific suppression indicates that although the mutants are each dimeric and suppressed by mutants that increase oligomerization, L66A has different defects than V143A and K142stop. Allele-specific suppression has been observed for mutations in other proteins that inhibit conformational changes, including FliM, the switch of the flagellar motor, and the chaperones GroEL and DnaK (34–36). While these are examples of intergenic suppression, intragenic suppression could occur through a similar mechanism in sHSPs. Our conjecture that the hydrophobic patch has an additional function not shared by the C-terminal extension could explain the failure of suppressors of V143A to restore L66A activity. However, it does not explain the converse allele specificity, since the oligomer-stabilizing effects of D80V and V108L would be expected to restore the deficiency of V143A. Therefore, we propose instead that sHSP dimers may adopt two structures and that V143A and K142stop stabilize one structure, whereas L66A stabilizes the other. Suppression would require destabilizing the occupied dimeric state.

The suppressor mutations increase the oligomeric stability not only of their parent dimers but also of WT Hsp16.6 (6) (Fig. 7). The increase in oligomeric stability of WT Hsp16.6 by the suppressor is consistent with the model that there are two dimeric states. A mutation that destabilized either dimer would increase the relative stability of the oligomer. Nevertheless, the effect of destabilizing one dimeric state on a mutant protein that occupies the other state might be undetectable, since the relative stability of the oligomer and the stabilized dimer would remain the same.

We cannot rule out the possibility that L66A or V143A destabilizes Hsp16.6 so that the dimer becomes misfolded and unable to reoligomerize. In this case, one or both of the dimeric species would be off-pathway in the sHSP mechanism. However, the dimeric sHSPs both have luc-protection activity, whereas a misfolded protein is likely to be nonfunctional. For example, the R120G mutant of αB-crystallin is an unstable protein that actually increases substrate aggregation (37). Furthermore, the suppressor mutations S2Y, N7Y, D80V, and V108L all increase the oligomeric stability of WT Hsp16.6 in addition to that of either L66A or V143A. If a mutant were significantly misfolded, its suppressors would act to refold the protein and would not be expected to increase the stability of WT Hsp16.6 oligomers under conditions where WT is already folded and active. Thus, the allele specificity is more easily explained as resulting from mutations that stabilize two con-
formationally distinct dimer structures that are also occupied by WT Hsp16.6.

In total, we have demonstrated that a mutation of the C terminus destabilizes the oligomer of *Synechocystis* Hsp16.6 but is not as deleterious as a mutation in the hydrophobic patch either in *vivo* or *in vitro*. There may be a difference in the functions of the two regions of the sHSP, despite a shared role in oligomerization. Based on observed allele specificity of suppressors, these mutations also appear to stabilize different dimeric conformations of Hsp16.6. The existence of two dimeric states of sHSPs has not been proposed previously. So little is known about the chaperone mechanism of sHSPs that it is not possible to predict the significance of dimers adopting multiple conformations. Since L66A and V143A are both able to complement the activity of the WT sHSP, we suggest that these two dimeric states each play a role in protection of misfolding proteins by sHSPs. These mutants should therefore be useful in further studies characterizing the structure and activity of sHSP dimers.

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Mutants in a Small Heat Shock Protein That Affect the Oligomeric State: ANALYSIS AND ALLELE-SPECIFIC SUPPRESSION

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