The ability of small heat shock proteins (sHSPs) to prevent thermal aggregation of other proteins may require disassembly and reassembly of sHSP oligomers. We investigated the role of changes in sHSP oligomerization by studying a mutant with reduced oligomeric stability. In HSP16.6, the single sHSP in the cyanobacterium Synechocystis sp. PCC 6803, the mutation L66A causes oligomer instability and reduced chaperone activity in vitro. Because thermotolerance of Synechocystis depends on HSP16.6, a phenotype that is enhanced in a ΔClpB1 strain, the effect of mutations can also be assayed in vivo. L66A causes severe defects in thermotolerance, suggesting that oligomeric stability of sHSPs is required for cellular function. This hypothesis was supported by a selection for intragenic suppressors of L66A, which identified mutations that stabilize oligomers of both L66A and wild-type HSP16.6. Analysis of both over- and under-oligomerizing mutants suggests that sHSPs must disassemble before they can release substrates. Furthermore, the suppressor mutations not only restore in vivo activity to L66A, they also ameliorate chaperone defects in vitro, and thus provide the first direct evidence for a chaperone function of an sHSP in cellular thermotolerance.

Molecular chaperones prevent irreversible damage to other proteins during heat stress. Most chaperones act to assist in protein folding, but small heat shock proteins (sHSPs) appear to be limited to maintaining the solubility of unfolding proteins, without catalyzing refolding (1). The mechanism for this protection is not known, but in vitro studies with model substrates have identified stable, soluble complexes between sHSP oligomers (typically 9–30 or more monomers) and their substrates (for review, see Ref. 2). According to current models, de-oligomerization is an essential step in sHSP function (3–5). Heat-induced destabilization of the sHSP oligomer may result in a smaller species that initiates the interaction with substrate, followed by re-assembly into a larger sHSP-substrate complex. Although sHSPs do not promote refolding of these model substrates themselves, sHSP-bound proteins have been refolded with ATP-dependent chaperones such as the HSP70 system or GroE (6, 7). How these biochemical activities relate to the action of sHSPs in vivo remains to be elucidated.

The crystal structures of two sHSPs are known. HSP16.5, a spherical, 24-subunit oligomer from Methanococcus jannaschii was crystallized by Kim et al. (8). Comparison with wheat TaHSP16.9, a dodecameric disk (5), suggests that a dimer will be a common building block of many sHSP oligomers. The ~100-amino acid α-crystallin domain, which is the region best conserved between sHSPs (9), contains the dimer interface. This domain forms a β-sandwich in which a β-strand of each monomer is incorporated into a β-sheet of the other. The α-crystallin domain is flanked by a variable length, nonconserved N terminus and a short, flexible C-terminal arm. Both high resolution structures reveal inter-dimer interactions between hydrophobic residues in the C-terminal arm (β-strand 10) with a hydrophobic patch on the surface of the α-crystallin domain (largely β-strands 4, 5, and 8). Both groups of hydrophobic residues in this interaction are highly conserved in all sHSPs (9). This interaction appears to be important for oligomeric stability, but its role in the chaperone activity of sHSPs is unknown.

sHSPs enhance stress tolerance in a variety of cell systems (10, 11), but are often nonessential for thermotolerance (12, 13). Three organisms have been shown to become heat-sensitive in the absence of an sHSP gene: Neurospora crassa (14), Synechocystis sp. strain PCC 6803 (15) (referred to hereafter as Synechocystis), and recently Escherichia coli (16). In these reports, the loss of viability of the sHSP deletions were mild, on the order of a 10-fold decrease compared with wild type, making these phenotypes difficult to exploit genetically. For this reason we undertook developing a more robust assay for sHSP activity in vivo that would allow selection for sHSP function and enable critical in vivo tests of the chaperone mechanism of sHSP action.

Synechocystis has many advantages for molecular studies. In addition to having a fully sequenced genome (17), it is easily transformed, and homologous recombination into the chromosome allows deletion and replacement of target genes (18). Therefore HSP16.6, the only sHSP in Synechocystis, can be deleted and replaced by mutant variants. In this study we describe a stress condition that demonstrates a strong requirement for functional HSP16.6, and allows the effects of point mutations on sHSP function in vivo to be assayed. Analysis of HSP16.6 in its homologous system may facilitate identification of mutants that disrupt in vivo function because of changes in essential, but as yet unrecognized activities of sHSPs. Synechocystis HSP16.6, which comprises relatively uniform, highly soluble oligomers, is also more readily studied in vitro than the analogous sHSPs from E. coli, which aggregate on purification (16). Thus Synechocystis presents the opportunity to correlate in vivo and in vitro activities of an sHSP.
We show here that mutations in HSP16.6 at Leu-66, a conserved residue in the hydrophobic patch on the α-crystallin domain, cause severe thermotolerance defects in Synechocystis. One of these mutant proteins, L66A, is also greatly impaired in both oligomerization and chaperone activity in vivo. In a novel selection for hSP function, we randomly mutated hsp16.6 L66A and selected for intragenic suppressors that restore hSP activity in vivo. This selection led to the identification of mutations that over-stabilize the HSP16.6 oligomer, and restore activity to the L66A mutant both in vivo and in vitro. The rate at which an hSP-protected substrate is refolded by reticulocyte lysate is affected both by mutants with reduced oligomeric stability, which increase the rate, and strongly oligomerized mutants which slow it. This suggests a requirement for hSP disassembly prior to substrate release. In total, these data demonstrate a correlation between hSP function in vivo and chaperone activity in vitro, and support the hypothesis that dynamic changes in oligomerization are essential to both.

**EXPERIMENTAL PROCEDURES**

**Plasmids—**pNaive (pAZ722) is a pUC118-based plasmid derived from pH-K28, for integration at the hsp16.6 locus (open reading frame sl1514 flank) by homologous recombination. pClpB1-KO (pAZ804) and pClpB2-KO (pAZ805) are deletion constructs for clpB1 and clpB2. The pBluescript (Stratagene)-based plasmids pClpB1-KO (pAZ804) and pClpB2-KO (pAZ805) are deletion constructs for clpB1 and clpB2, respectively (17). Each contains 500 bp of upstream and downstream flanking sequences from either clpB gene (generated by PCR on wild-type genomic DNA), separated by an erythromycin resistance gene from pLAI25 (19).

pC20/Hpa (pAZ677) was created from pC20 (20) by adding an HpaI site to the polylinker. This allowed hsp16.6 to be inserted using HpaI and Apal to make pC20/Hpa (pAZ730).

**Synechocystis Strains—**All strains in this work were created by transforming pNaive into hsp16.6 deletion cells, to ensure that recombination occurs outside of the hsp16.6 gene. The isogenic ΔHSP16.6 and ΔHSP16.6 strains were made by transforming pNaive and pNaive.16 into HK-1, a kanamycin-resistant, hsp16.6 deletion strain, provided by Drs. Kosaka and Fukuzawa of Kyoto University. Transformations were done as described by Williams (21), selecting for increasing spectinomycin resistance, at concentrations up to 250 μg/ml spectinomycin plates.

Initial ClpB deletion strains were made by transforming pClpB1-KO and pClpB2-KO into both +HSP16.6 and ΔHSP16.6, and selected for with up to 300 μg/ml erythromycin. pClpB1-KO was also transformed into HK-1 cells to create ΔClpB1/HK-1, which was used as the parental strain in most experiments. pNaive vectors carrying the appropriate hsp16.6 alleles were transformed to make +HSP16.6/ΔClpB1, ΔHSP16.6/ΔClpB1, and other mutant strains. Experiments were performed with at least two independent transformants for each strain.

**Synechocystis Growth Conditions—**Cells were maintained in a lit 30 °C incubator on BG-11/sugar (22) plates, buffered with 10 mM TES, pH 8.2, supplemented with 5 mM glucose, and either 50 μg/ml kanamycin sulfate, 100 μg/ml spectinomycin dihydrochloride, or 100 μg/ml erythromycin sulfate, as appropriate. Liquid media was BG-11, buffered with 5 mM HEPES, pH 7.8, supplemented with 5 mM glucose, and did not contain antibiotics. Suspension cultures were grown on a rotator wheel at 30 °C, resulting in doubling times of ~8 h, and maximum cell densities of OD730 ~2.5. Care was taken to ensure cells were in early log phase prior to stress treatments. Changes at the hsp16.6 and clpB1 loci did not affect cell growth rates or maximum densities prior to heat stress.

**Heat Shock Assays—**Liquid cultures of logarithmically growing cells were diluted to an OD730 of 0.07 20 h before the stress. On the day of the experiment, densities were typically 0.3–0.6 OD730. Cultures were all diluted with fresh media to OD730 = 0.25, and serially diluted 1:10 four times, and were incubated at 42 °C in a Thermo Hi Performance incubator (Precision). Colonies typically appeared within 6 days. Survival was determined by comparing the number of colonies on heat-treated plates with unheated, BG-11/glucose-only plates.

**Site-directed Mutagenesis—**The hsp16.6 Leu-66 mutants were created with PCR using pC20/Hpa.hsp16 as a template, and 5′- phosphoribylated oligonucleotides designed to randomly mutate the Leu-66 codon. A pair of oligos was designed so that each annealed to opposite strands, and their 5′ ends annealed to adjacent nucleotides. PCR was performed with Pfu Turbo (Stratagene), and resulted in a linearized plasmid that could be circularized by ligating its blunt ends. These plasmids were amplified in E. coli. hsp16.6 was sequenced before being subcloned into pNaive. These plasmids were transformed into the HK-1/ΔclpB1 strain. This same procedure was used for all site-directed mutagenesis.

**Random Mutagenesis—**Mutagenesis of hsp16.6 L66A was done using error-prone PCR with Taq polymerase (Roche) in the presence of MnCl2, as described by Leung et al. (23). pNaive.16 L66A (pAZ697) was used as a template. The oligos anneal on either side of the hsp16.6 gene, amplifying the entire gene. Buffer conditions were as directed by Roche for Taq polymerase, except that there was 0.1 mM MnCl2, 4.9 mM MgCl2, and 80 μM dNTPs. 30 cycles of amplification were performed. Under these conditions, we estimated an average of ~1.5 base pair changes/gene, and found a range from 0 to 6. Resulting PCR fragments were dialyzed with Hpad and ApalI and cloned into pNaive as described above. Pools of plasmids were amplified in E. coli before transforming into Synechocystis.

**Determination of HSP16.6 Accumulation—**Quantitation of logarhythmically growing cells were incubated in a 14 °C water bath for 2 h, and then pelleted at 4 °C before being resuspended in SDS sample buffer. The protein concentration of the cell lysates was measured with Coomassie Blue binding (24). 0.5 μg of protein/lane was loaded on 15% SDS-PAGE gels. Western blot analysis was performed with anti-HSP16.6 antisemur, created against purified recombinant HSP16.6.

**Selection for hSP Function—**Plaoids of plasmids containing randomly mutagenized hsp16.6 L66A were transformed into HK-1/ΔclpB1. 3000 mutagenized genes from 10 independent PCR reactions were transformed as described above, except that cells were replica-plated to 250 μg/ml spectinomycin plates, and then 7 days later to drug-free plates. Four days later they were again replica-plated to 20 ml, 140 mM MgSO4 BG-11/glucose plates, and heated at 44 °C for 8 h. Plates were moved to 30 °C, and allowed to grow for 8–10 days. By this time, large patches of cells were observed from surviving colonies. The hsp16.6 genes were amplified out of potential suppressor strains and sequenced. To ensure that the observed phenotype was hsp16.6-dependent, the genes were then re-transformed into Synechocystis, and cells were re-selected for their heat stress sensitivity.

**Protein Purification—**HSP16.6 and its mutant versions were purified as previously described (25). Proteins were expressed from pC20/Hpa plasmids in the E. coli strain BL21 (Stratagene). Unlike the wild-type HSP16.6, L66A and L66A/D80V were in the insoluble fraction of the lysate and were resolubilized with 6 M urea. When the urea was dialyzed away, the HSPs remained soluble. Similar treatment of wild-type protein had no effect on its activity or oligomerization. L66A and L66A/D80V were insoluble in low concentrations of ammonium sulfate; therefore, this step of the purification was omitted for them. The 0.2–0.85 M sucrose gradient, and the ion exchange on DEAE in 3 M urea were the same for all samples. Proteins were stored in 20 mM NaPO4, 20 mM NaCl, pH 7.3, 1 mM dithiothreitol.

**Protein concentration of HSP16.6 was determined using an extinction coefficient of ε280 = 5960 M−1 cm−1, based on the aromatic amino acid content, as described by Pace et al. (26).** Mutant proteins were assayed by Bradford assay (27), using HSP16.6 as a standard.

**Size Exclusion Chromatography (SEC)—**Proteins were run on a Bio-Sephacel S200 (Bio-Rad), equilibrated with 20 mM sodium phosphate, pH 7.3, 0.1 mM dithiothreitol.

**Protein concentration of HSP16.6 was determined using an extinction coefficient of ε280 = 5960 M−1 cm−1, based on the aromatic amino acid content, as described by Pace et al. (26).** Mutant proteins were assayed by Bradford assay (27), using HSP16.6 as a standard.

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1. H. Kosaka and H. Fukuzawa, unpublished data.
2. H. Kosaka and H. Fukuzawa, unpublished data.
3. J. Lee and E. Vierling, unpublished data.
sHSP Activity Requires Changes in Oligomerization

RESULTS

Assay for sHSP Function in Synechocystis—We sought conditions that require functional HSP16.6 for survival in a simple plating assay. A variety of stress conditions were tested, and a combination of MgSO₄ and 44 °C heat stress was determined to best demonstrate sHSP-dependent survival. Fig. 1A shows the isogenic strains +HSP16.6, a wild-type HSP16.6-expressing strain, and ΔHSP16.6, an hsp16.6 deletion strain, plated onto standard agar plates or plates supplemented with 140 mM MgSO₄. In the absence of heat stress, there is no loss of viability by either strain on MgSO₄. When heated for 8 h at 44 °C on MgSO₄, less than 0.1% of ΔHSP16.6 survive compared with greater than 10% of +HSP16.6. Thus, the deletion of the sHSP causes more than 100-fold loss of viability.

Enhanced Dependence on HSP16.6 in ΔClpB1 Cells—The ClpB/HSP100 proteins are a family of chaperones that have the ability to resolubilize aggregated proteins (28–30). The loss of sHSP function, which might lead to increased protein aggregation, could be compensated for by the action of ClpB. A search of the Synechocystis data base, Cyanobase (www.kazusa.or.jp/cyano), identified two clpB genes (slr1641 and slr0156) that we have named clpB1 and clpB2, respectively, based on the similarity of the former to the heat-induced clpB1 in Synechococcus sp. strain PCC 7942 (31). clpB1 deletions were readily obtained in both +HSP16.6 and ΔHSP16.6 backgrounds with no effect on cell growth at 30 °C. Parallel attempts to delete clpB2 were unsuccessful in both strains, suggesting that, as was found in Synechococcus (32), this gene is essential under standard growth conditions.

As shown in Fig. 1, there are not significant differences in the survival of the +HSP16.6 and +HSP16.6/ΔClpB1 strains after heat shock. However, in the ΔClpB1 background, the hsp16.6 deletion, ΔHSP16.6/ΔClpB1, is >10,000-fold less viable than +HSP16.6/ΔClpB1. Our data are suggestive, but do not prove, that there is a genetic interaction between these proteins. Nevertheless, because of the strong dependence of Synechocystis thermotolerance on HSP16.6 in the absence of ClpB1, all selections and subsequent analyses were performed in ΔClpB1 cells.

Mutation of a Conserved Hydrophobic Residue of HSP16.6 Causes a Thermosensitivity Greater than ΔHSP16.6—As described in the Introduction, several conserved hydrophobic amino acids form a patch on the surface of sHSPs that may be an important oligomerization site. We wished to test the importance of sHSP oligomerization on in vivo function by mutating one of these conserved residues in HSP16.6, and examining the effect on thermotolerance in Synechocystis. Leu-66, on β-strand 4, was chosen because mutagenesis of a homologous residue, Val-76 in Pismum sativum HSP18.1, was found to disrupt the sHSP oligomer in vitro.

Transformation of Leu-66 mutant alleles into a ΔHSP16.6/ΔClpB1 background (described under “Experimental Procedures”) results in expression of these mutants by the endogenous hsp16.6 promoter in the absence of wild-type HSP16.6. As shown in Fig. 2A, mutations of Leu-66 have varied effects. L66T has little effect on thermotolerance, whereas L66E and L66K mutant proteins are greatly reduced relative to wild-type HSP16.6 in vivo. L66A, L66E, and L66K mutant proteins are less viable than the deletion strain. Even cells carrying the conservative mutation L66A are nearly as defective as ΔHSP16.6/ΔClpB1, demonstrating that small changes at Leu-66 can greatly impair HSP16.6 function in vivo.

The accumulation of HSP16.6 was measured by Western blot after a nonlethal incubation at 42 °C (Fig. 2B). The levels of L66A, L66E, and L66K mutant proteins are greatly reduced relative to wild-type HSP16.6, suggesting either that they are unstable or that they are degraded because their presence is deleterious to the cell. Even L66T-expressing cells, which are wild type in survival, do not accumulate wild-type levels of sHSP, indicating that cells with reduced levels of sHSP can remain thermotolerant.

Identification of Intragenic Suppressors of hsp16.6 L66A—Intragenic suppressor analysis was undertaken to identify regions of HSP16.6 that share their function with Leu-66.

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4 D. S. Kim and E. Vierling, unpublished data.
HSP16.6 prior to heat treatment (data not shown).

described under “Experimental Procedures.” Strains show standard deviation.

Fig. 2. Heat stress sensitivity of strains with mutations of Leu-66 in HSP16.6. A, viability of strains containing point mutants of Leu-66 in hsp16.6 compared with wild-type HSP16.6 and ΔHSP16.6 strains (all in ΔclpB1 background) after 8 h at 44 °C, as described in Fig. 1. Each bar represents the average of three to six samples; error bars show standard deviation. B, accumulation of HSP16.6 was determined by Western blot of lysates of cells treated at 42 °C for 2 h, as described under “Experimental Procedures.” Strains show negligible HSP16.6 prior to heat treatment (data not shown).

The severe reduction of thermostolerance of cells carrying hsp16.6 L66K made this mutation appear to be an excellent tool to isolate suppressors that would restore sHSP function in vitro. However, multiple attempts to identify suppressors of L66K failed, suggesting that it may be too severe to suppress in the manner tried. In contrast, suppressors of the weaker mutant, L66A, were readily obtained.

Intragenic suppressors were generated by random mutagenesis of hsp16.6 L66A by error-prone PCR and transformed into a Synechocystis Δhsp16.6 ΔclpB1 strain. The hsp16.6 genes of colonies that survived 44 °C 8 h were recovered and sequenced. Mutant genes were re-transformed into Synechocystis to verify that thermostolerance was sHSP-dependent. Eight suppressors were isolated (Table I), representing single amino acid changes at five residues, and one double mutant (P8L/K137E) out of ~3000 colonies screened. Three changes at Asp-80 (to Val, His, or Asn) all suppress the L66A defect. L66A/N40Y has been independently isolated three times, suggesting that this selection is approaching saturation. The back mutation, Ala-66 to Leu, was not recovered, but this mutation is unlikely as it would require two base changes (GCC to either CTG or TTG). Ala-66 to Thr (ACG), which can substitute for Leu-66 (Fig. 2A), was recovered.

The ability of the suppressors to restore thermostolerance is shown in Fig. 3A. Some suppressors, such as N40Y and V108L, are strong enough to rescue L66A to nearly wild-type levels of survival, whereas L66A/V133A is just 10-fold better than L66A alone. Suppression by P8L and K137E individually has also been tested. Each mutation can at least slightly suppress L66A, although neither does as well as P8L/K137E.

Some of the suppressor mutations improve HSP16.6 accumulation. Fig. 3B shows HSP16.6 levels in cells expressing the suppressor mutants relative to wild-type and L66A-expressing strains. None of the suppressors is able to fully restore wild-type levels of HSP16.6, and some accumulate little more than L66A. Thermostolerance does not correlate well with sHSP accumulation. For example, L66A/N40Y survives better than L66A/D80V, but accumulates less sHSP.

Suppressor Mutations Alone Have No Effect on Thermostolerance—The suppressors of L66A have the potential to impair sHSP function in the absence of the L66A mutation. To test this, the thermostolerance of cells expressing hsp16.6 genes carrying only the suppressor mutations has been measured. Cells expressing any of these suppressor-only mutants survive 8 h at 44 °C as well as wild type (Table I).

It is possible that these mutations have slight defects that the thermostolerance assay is not sensitive enough to measure. Reasoning that small defects of the suppressors might be additive in a double mutant, N40V/D80V and D80V/V108L were constructed and transformed into Synechocystis to look for an effect on heat stress survival. Both of these resulting strains have wild-type thermostolerance. Therefore we conclude that the suppressor mutants do not significantly affect HSP16.6 function in this assay.

Suppressors of L66A Restore sHSP Oligomerization—Having identified suppressors of L66A, we examined their effects on known biochemical properties of HSP16.6 to compare their effects on in vivo and in vitro function. Fig. 4 shows the relative size of HSP16.6 mutant proteins, purified from E. coli, as determined by SEC. The L66A oligomer is less stable than wild type, even at room temperature (solid lines). Under conditions where wild-type HSP16.6 elutes as a single species, which is ~400 kDa, consistent with an oligomer on the order of 24 monomers, ~20% of L66A appears to be 40–50 kDa, consistent with an sHSP dimer or trimer. Increasing the concentration of L66A from 24 to 96 μM (Fig. 4B) decreases the fraction of protein in the suboligomeric state, but does not eliminate it.

Because sHSPs bind proteins denatured at elevated temperature, the effects of heat treatments on the oligomeric structure of the mutants were examined. When 24 μM L66A is heated (42 °C for 7.5 min) and then cooled (4 °C for 20 min) before being injected onto the column, nearly all of it is found in the small form (Fig. 4A, dashed line). In contrast, wild-type HSP16.6 is only slightly destabilized by this heat treatment. The species made by heating L66A is very stable, because a similar profile was observed when the sample was injected onto the column 24 h after heating (thin, dotted line). At 96 μM, L66A is still destabilized by heat, but shows better restoration of oligomerization after 24 h than it does at 24 μM, indicating that de-oligomerization of L66A is reversible, and re-oligomerization is concentration-dependent.

The mutations D80V and V108L suppress the oligomerization defect of L66A (Fig. 4A). However, oligomers of L66A/D80V and L66A/V108L elute slightly later than wild-type HSP16.6, and the differences are increased when the proteins are heated and cooled. Heating destabilizes both L66A/D80V and L66A/
Table I

| Suppressor mutant(s) | Independent isolates (no.) | Increase in hydrophobicity | Thermotolerance of L66A with suppressor* | Thermotolerance of suppressor alone^ |
|----------------------|----------------------------|---------------------------|----------------------------------------|-----------------------------------|
| N40Y                 | 3                          | 6.5                       | +++                                    | +++                                 |
| T76I                 | 1                          | 7.5                       | +++                                    | +++                                 |
| D80V                 | 1                          | 12.8                      | +                                      | +++                                 |
| D80H                 | 1                          | 4.1                       | +                                      | +++                                 |
| D80N                 | 1                          | 2.1                       | +                                      | ND                                 |
| V108L                | 1                          | 0.9                       | +                                      | +++                                 |
| V133A                | 2                          | –2.2                      | +                                      | +++                                 |
| P8L/K137E            | 1                          | ND                        | +                                      | +++                                 |
| P8L                 | ND                         | –1.3                      | +                                      | ++                                  |
| K137E               | ND                         | –1.3                      | +                                      | ++                                  |

* As calculated by Radzicka and Wolfenden (38) for side-chain analogs, in kcal/mol. The hydrophobicity of proline was not determined.
^ Scale is “+++” = wild-type thermotolerance, “–” = L66A.
^ ND, not determined.

Chaperone Activity of HSP16.6 Mutants—Like other sHSPs, HSP16.6 protects model substrates from aggregation in vitro (33). The ability of L66A and its suppressors to maintain the solubility of luc was compared with wild type. HSP16.6 can fully protect luc from becoming insoluble at a ratio of 1 μM luc to 24 μM sHSP (Fig. 6A). At this concentration, L66A is not able to protect luc; the amount of soluble luc in the presence of L66A is little better than the no sHSP control. More protection was observed when 1 μM luc was heated with 96 μM L66A, although nearly half the luc was still insoluble (Fig. 6B). When the concentration of luc was increased to 4 μM, the amount of luc protected by 96 μM L66A was the same as shown in Fig. 6B (data not shown). This indicates that the defect of L66A is not its affinity for substrate. Instead, L66A is impaired in its capacity for substrate and requires more of the mutant sHSP to prevent substrate aggregation.

The ability of L66A to prevent aggregation of luc is restored by the suppressor mutations. The double mutants L66A/D80V and L66A/V108L protect 1 μM luc from aggregation as well as wild type, at both 24 and 96 μM. The same is true for the suppressors, D80V and V108L, alone.

To characterize the chaperone activity of these proteins further, we measured the reactivation of sHSP-protected luc by ATP-dependent chaperones in reticulocyte lysate. As shown in Fig. 7, after heating 1 μM luc with 24 μM sHSP, luc was restored to ~70% of its pre-heated activity, but only to 5% when heated with an equivalent weight of bovine serum albumin (BSA) instead. The amount of refolding increased only slightly, from 71 ± 6 to 81 ± 2%, by increasing HSP16.6 to 96 μM, demonstrating that 24 μM wild type is near saturation for protection of 1 μM luc.

Consistent with its aggregation, only 9.7 ± 0.2% of the 1 μM luc heated in the presence of 24 μM L66A can be reactivated. However, unlike BSA, L66A promotes significantly more luc reactivation at higher concentrations. At 96 μM L66A, luc re-activation increased to 66%, substantially more than would be expected if protection by L66A was linear with sHSP concentration. Further improvement in chaperone capacity has been observed at higher concentrations, but even at 480 μM L66A protects significantly less luc than does wild type at 24 μM, on a molar basis. Nevertheless, it is clear that L66A can maintain luc in a refoldable state.

Luciferase protected by either L66A/D80V or L66A/V108L can be nearly completely reactivated. In fact, at 24 μM the double mutants allowed the refolding of slightly more luc than wild-type HSP16.6. Thus, in addition to improving oligomerization of L66A, the suppressor mutations have fully restored in vitro chaperone activity to this mutant.

Although luc is maintained in a soluble state by D80V and V108L (Fig. 6), refolding of this protected protein is impaired.
When heated with 24 µM D80V or V108L, luc reactivation is significantly less than if heated with wild type. This suggests that some requirement for reactivation may be inhibited by the increased oligomeric stability of these mutants, although alternatives that are independent of oligomerization cannot be ruled out.

The rate of luc reactivation is significantly faster for the L66A/D80V and L66A/V108L protected samples (Fig. 7B). Lu-
FIG. 5. Oligomers of D80V and V108L are more stable than wild-type HSP16.6 at 38 °C. SEC was performed at 38 °C. 6 μM wild type (circles), D80V (squares), or V108L (triangles) were injected onto a column after being heated at 38 °C for at least 20 min. Arrowheads show the elution time of the oligomer (O) and of the suboligomeric species made by L66A (D).

FIG. 6. Protection of luc from aggregation by HSP16.6. Luciferase was heated at 42 °C for 7.5 min in the absence or presence of HSP16.6 before being centrifuged. Equal volumes of the soluble fraction were run on a 14% SDS-PAGE gel, and Coomassie-stained. A, 1 μM luc with 24 μM sHSP; B, 1 μM luc with 96 μM sHSP. Samples were compared with the amount of soluble luc in the unheated samples.

The mechanism of sHSP chaperone activity is poorly characterized, but is hypothesized to involve temperature-induced rearrangement of the sHSP oligomer. As suggested by Haslbeck et al. (3), a suboligomeric particle may act as the primary substrate-binding species, followed by re-assembly into a larger complex with the substrate. This hypothesis has supporting evidence from in vitro studies (5, 34), but is untested in heat-stressed cells. To examine the biological relevance of this model, we have examined the effect of altering the oligomeric stability of HSP16.6 on its in vitro function.

As described in the Introduction, the interaction between a conserved hydrophobic patch on the α-crystallin domain and the C-terminal arm of sHSPs has been suggested to be important for oligomerization. We have mutated a residue in the Leu-θ66 patch, Leu-66 in HSP16.6, and tested the effects on sHSP-dependent survival of heat stress. Whereas changes at this residue had varied effects, even the relatively conservative mutation L66A caused severe loss of HSP16.6 function in vivo. The L66A mutation destabilizes the HSP16.6 oligomer and leads to severe loss of chaperone activity in vitro. When transiently heated, L66A almost entirely de-oligomerized into a single suboligomeric species. It is tempting to speculate that the suboligomeric state observed is an sHSP dimer, as the crystal structures suggest that dimers are the most stable suboligomeric form (5, 8). However, other suboligomeric species cannot be ruled out by SEC analysis. The in vitro chaperone activity of L66A is also impaired, so that at 24 μM, which is sufficient for function of wild-type HSP16.6, L66A can neither maintain 1 μM luc in a folding-resistant state, nor prevent luc aggregation.

The failure of L66A to protect luc from aggregation may be the result of a deficiency in assembly of a normal sHSP-substrate complex. The protection of luc by L66A was improved by increasing the sHSP concentration, but not by increasing the concentration of luc, demonstrating that the defect is the capacity, not the affinity, of L66A for luc (Fig 6). These data suggest that L66A is defective in a cooperative association with itself that is essential for efficient protection of substrate. This could be a cooperative assembly of dimers into an sHSP-substrate complex. Little is known about the structure of these sHSP-substrate complexes, but their assembly may require some of the same contacts between sHSP dimers as are used for...
oligomerization in the absence of substrate. Thus, assembly of complexes, in addition to oligomerization, could be impaired by the L66A mutation. Attempts to observe complexes between L66A and luc by SEC have failed, although this negative result could be caused by instability of complexes rather than by their absence.

Although L66A does not function as efficiently as wild type, at 96 μM it does protect 0.6 μM luc from aggregation. At this concentration we have observed that directly after being heated L66A is nearly all suboligomeric. L66A may act through interactions. The three weakest suppressors, P8L, V133A, and K137E, do not map to this proposed interface, and the significance of their locations is not known.

We used a thermotolerance assay to select for second-site suppressor mutations of the hsp16.6 L66A mutant gene, to identify regions of HSP16.6 that share the function of Leu-66. The hypothesis that the oligomerization defect of L66A is responsible for its failure in vivo predicted that suppressor mutations would identify other residues involved with oligomerization, whereas other possible mechanisms for its loss of function would require different suppressors. Little is known about the functional domains of sHSPs, and so such structure-function data are desirable. This approach should also be applicable to other types of sHSP mutants, such as mutants impaired in substrate binding, to map different functional regions of HSP16.6.

We have identified seven residues that can be mutated to restore function to L66A in vivo. The location of equivalent residues in the structure of MjHSP16.5 (8) is shown in Fig. 8. V108L is the only suppressor in the conserved hydrophobic patch with Leu-66 (Fig. 8A). V108L might stabilize the arm/patch interaction by increasing the hydrophobicity of the patch, thus directly reversing the effect of L66A. Although theoretically possible, none of the suppressors increases the hydrophobicity of the C-terminal arm.

We suggest that five of the suppressors define an oligomerization interface for HSP16.6. N40Y, T76I, D80H, D80N, and D80V map onto one surface of the β-sandwich formed by the α-crystallin domain, on the turn before β-strand 2, and on β5, and all point away from the dimer (Fig. 8B). These mutations increase the hydrophobicity of this face (Table I), and might therefore be expected to favor oligomerization. This genetically defined oligomerization interface of HSP16.6 is not an obvious prediction of the oligomeric structure of either MjHSP16.5 or TaHSP16.9, although in MjHSP16.5 the residue equivalent to HSP16.6 Thr-76 is in contact with an adjoining dimer. How hydrophobicity of this face (Table I), and might therefore be expected to favor oligomerization. This genetically defined oligomerization interface of HSP16.6 is not an obvious prediction of the oligomeric structure of either MjHSP16.5 or TaHSP16.9, although in MjHSP16.5 the residue equivalent to HSP16.6 Thr-76 is in contact with an adjoining dimer. However, the oligomeric structures of sHSPs vary greatly (2), making it unlikely that they will share all of the same oligomeric interactions. The three weakest suppressors, P8L, V133A, and K137E, do not map to this proposed interface, and the significance of their locations is not known.

The suppressors of L66A give us insight into the nature of the defect caused by the mutation, namely that loss of oligomerization is the cause for the loss of chaperone activity. If the oligomerization defect of L66A was irrelevant to in vivo function, suppressors would be unlikely to restore this property. The second-site mutations D80V and V108L suppress both oligomerization and chaperone defects of L66A (Table II), although, as described above, the two mutations probably

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**Table II**

| Protein     | Thermotolerance | Oligomer stability | Luciferase solubility | Luciferase reactivation |
|-------------|-----------------|--------------------|-----------------------|-------------------------|
| Wild type   | +++             | +++                | +++                   | 71 ± 6 27              |
| L66A        | -               | +                  | -                     | 9.7 ± 0.2 <10          |
| L66A/D80V   | + +             | +                  | +++                   | 80 ± 4 <10             |
| L66A/V108L  | + +             | ++                 | +++                   | 80 ± 6 <10             |
| D80V        | + +             | ++                 | +++                   | 52 ± 7 32              |
| V108L       | +++             | +++                | +++                   | 47 ± 5 40              |

a Viability of mutant strains relative to wild-type = “+++.”

b Thermotolerance assay relative to wild-type HSP16.6 = “+++.”

c Estimated on experiments performed with 1 μM luc heated with 24 μM sHSP.

d Yield” and “−t1/2” represent the luciferase reactivation.

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![Figure 8](image-url)  
**Fig. 8. Predicted location of mutants on sHSP dimer structure.** The ribbon structure of a dimer (green and gray) of MjHSP16.5 (8) with the C-terminal arm from another dimer shown in orange. A, view of outer surface of oligomer, so that inside of oligomer is within the page. Hydrophobic patch is space-filled in white, with L66A in pink. B, the structure in A has been rotated forward 90° to show top, and the postulated oligomerization interface. Residues analogous to those of SynHSP16.6 found as suppressors of L66A are space-filled in blue, and labeled with the HSP16.6 amino acids and numbers. This figure was made using SwissPdb Viewer (37).
strengthen different oligomerization interfaces. The increase in oligomerization by these suppressor mutations is strong evidence that the oligomerization defect of L66A is integral to its loss of function.

There is an excellent correlation between the function of the HSP16.6 mutants in vivo and their ability to suppress luc aggregation in vitro, implying that this activity is essential to in vivo function. However, there are some differences between which proteins work best in vivo and in the luc reaction assay (Table I). At 24 μM, both L66A/D80V and L66A/V108L protected slightly more luc in a folding-competent state than wild type, whereas the in vivo thermotolerance provided by L66A/D80V is roughly 4-fold less than wild type. The single mutant D80V appears to be as functional as wild-type HSP16.6 in vivo, but is worse in the luc reaction assay. These differences may simply reflect the very different conditions between an 8-h heat stress in a cell compared with heat denaturation of purified proteins in less than 8 min, or differences in the sensitivities of the two assays. However, it is also possible that they reflect real discrepancies between what we know sHSPs are capable of doing in vitro, and their actual functions in vivo. An advantage of our genetic assay is that it makes no assumptions about what activities are important for sHSP function in vivo.

There appears to be an inverse relationship between the oligomeric stability of an sHSP and the rate of sHSP-protected luc refolding by ATP-dependent chaperones. The mutants D80V and V108L form oligomers that are more stable than wild type and slow the rate of luc refolding by reticulocyte lysate. In contrast, the mutants that make less stable oligomers, L66A/D80V, L66A/V108L, and L66A (at high concentration), allow reticulocyte lysate to refold luc very rapidly (Table I). We suggest that a step that is normally rate-limiting for substrate release from sHSPs has been accelerated in these mutants that are reduced in oligomerization, and that this same step is slowed in the over-oligomerized mutants. One simple model for how oligomerization could be related to the rate of substrate release is if disassembly of hSP dimers from the sHSP-substrate complex were an essential step in substrate release. It will be necessary to develop quantitative assays of substrate release to test this model. Although substrate release has been proposed as the rate-limiting step of malate dehydrogenase refolding from IpH3 (36), it has never been directly observed. Mutations like V108L and D80V, which inhibit substrate refolding, should be useful tools for investigating this step in the chaperone mechanism.

In conclusion, analysis of mutants of HSP16.6 provide the first direct, in vivo support for the chaperone model of sHSP function and demonstrate that changes in oligomerization are essential to chaperone activity. Mutations that change oligomeric stability should allow study of functional intermediates, that have, until now, been too short-lived to define. The ability to use the sHSP from Synechocystis for both genetics and biochemical analysis affords new opportunities for dissecting sHSP function.

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Note Added in Proof—The sHSP concentrations given are double those originally stated in Papers in Press.

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Kim C. Giese and Elizabeth Vierling

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