THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 48, Issue of November 29, pp. 46310–46318, 2002
Printed in U.S.A.

Changes in Oligomerization Are Essential for the Chaperone Activity of a Small Heat Shock Protein *in Vivo* and *in Vitro**

Received for publication, August 30, 2002, and in revised form, September 20, 2002
Published, JBC Papers in Press, September 23, 2002, DOI 10.1074/jbc.M208926200

Kim C. Giese and Elizabeth Vierling‡

From the Department of Biochemistry and Molecular Biophysics, University of Arizona, Tucson, Arizona 85721

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)[1] 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.

* This work was supported by National Institutes of Health (NIH) Fellowship F32 GM18966 (to K. C. G.) and NIH Grant RO1 GM42762 (to E. V.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biophysics, University of Arizona, 1007 E. Lowell St., Tucson, AZ 85721. Tel.: 520-621-1601; Fax: 520-621-3709; E-mail: vierling@email.arizona.edu.

[1] The abbreviations used are: sHSP, small heat shock protein; BSA, bovine serum albumin; SEC, size exclusion chromatography; luc, firefly luciferase; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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 vitro*. In a novel selection for sHSP function, we randomly mutated *hsp16.6 L66A* and selected for intragenic suppressors that restore sHSP 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 sHSP-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 sHSP disassembly prior to substrate release. In total, these data demonstrate a correlation between sHSP 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 pHK-2R,[2] for integration at the *hsp16.6* locus (open reading frame sll1514 (Ref. 17)) via flanking sequence (500 bp each) from both ends of the *hsp16.6* gene. Using unique restriction sites (*Hpa*I, found in the *hsp16.6* promoter just upstream of the start codon, and an engineered *Apa*I site after the stop codon), *hsp16.6* was cloned into pNaive to make pNaive.16 (pAZ768). The spectinomycin resistance gene, *aadA*, is 150 bp downstream of the *hsp16.6* stop codon.

The pBluescript (Stratagene)-based plasmids pClpB1-KO (pAZ804) and pClpB2-KO (pAZ805) are deletion constructs for *clpB1* and *clpB2*, *Synechocystis* genes *slr1641* and *slr0156*, 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 pRL425 (19).

pJC20/Hpa (pAZ877) was created from pJC20 (20) by adding an *Hpa*I site to the polylinker. This allowed *hsp16.6* to be inserted using *Hpa*I and *Apa*I to make pJC20/Hpa.hsp16 (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 +HSP16.6 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 dihydrochloride.

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/agar (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 rotating wheel at 30 °C, resulting in doubling times of ~8 h, and maximum cell densities of OD_{730} ~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 OD_{730} of 0.07 20 h before the stress. On the day of the experiment, densities were typically 0.3–0.6 OD_{730}. Cultures were all diluted with fresh media to OD_{730} = 0.25, and serially diluted 1:10 four times. Spots (5 μl) were applied to 20.0 (± 0.2)-ml BG-11/glucose plates, with or without 140 mM $MgSO_4$, as stated in the text. Plates were incubated either at 30 °C, or at 44 °C for up to 8 h in the dark in a Thelco 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 pJC20/Hpa.hsp16 as a template, and 5′-phosphorylated 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 $MnCl_2$, 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 $MnCl_2$, 4.9 mM $MgCl_2$, 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 digested with *Hpa*I and *Apa*I and cloned into pNaive as described above. Pools of plasmids were amplified in *E. coli* before transforming into *Synechocystis*.

Determination of HSP16.6 Accumulation—Liquid cultures of logarithmically growing cells were incubated in a 42 °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 rabbit antiserum, created against purified recombinant HSP16.6.[3]

Selection for sHSP Function—Pools 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 $MgSO_4$ 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-assayed for their heat stress sensitivity.

Protein Purification—HSP16.6 and its mutant versions were purified as previously described (25). Proteins were expressed from pJC20/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 sHSPs 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 $NaPO_4$, 20 mM NaCl, pH 7.3, 1 mM dithiothreitol.

Protein concentration of HSP16.6 was determined using an extinction coefficient of ϵ_{280} = 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-Sil SEC 400 (Bio-Rad), equilibrated with 20 mM $NaPO_4$, 20 mM NaCl, pH 7.3, at a flow-rate of 1 ml/min. Unless otherwise stated, both buffer and column were at room temperature. For high temperature experiments, both column and buffer were heated to 38 °C, and samples were incubated at appropriate temperature for at least 20 min before being injected onto column. Proteins were diluted into the same buffer and, when appropriate, heated at 42 °C for 7.5 min before centrifuging at 16,000 × *g* for 15 min at 4 °C. 100 μl of sample were injected onto the column.

[2] H. Kosaka and H. Fukuzawa, unpublished data.

[3] G. J. Lee and E. Vierling, unpublished data.

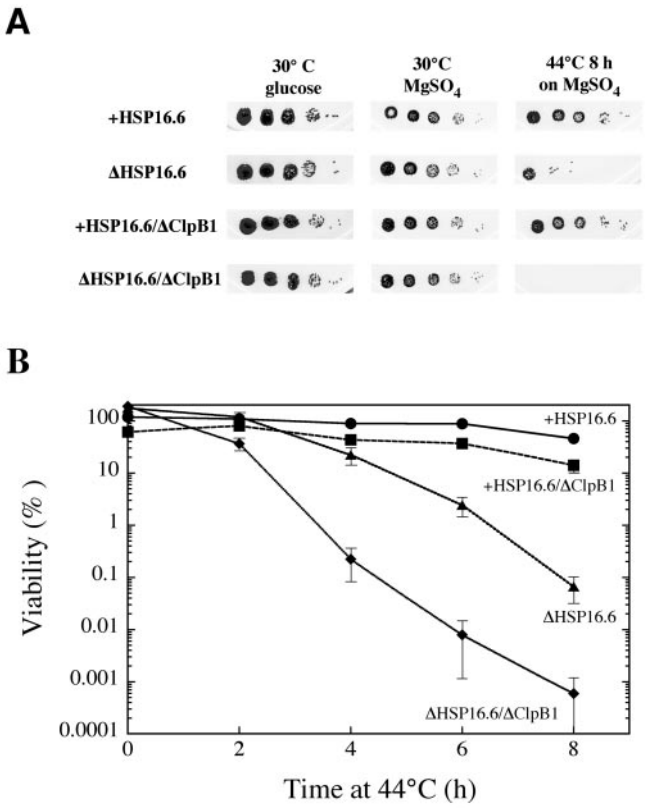

FIG. 1. **HSP16.6-dependent survival of heat stress.** *A*, the survival of 10-fold serially diluted cells of +HSP16.6, ΔHSP16.6, +HSP16.6/ΔClpB1, and ΔHSP16.6/ΔClpB1 strains grown at 30 °C on BG-11/glucose plates with or without 140 mM $MgSO_4$, or heat-stressed on 140 mM $MgSO_4$ at 44 °C for 8 h. *B*, time course of survival of 44 °C heat stress on 140 mM $MgSO_4$ plates. *Symbols* represent +HSP16.6 (*circles*), +HSP16.6/ΔClpB1 (*squares*), ΔHSP16.6 (*triangles*), and ΔHSP16.6/ΔClpB1 (*diamonds*). Each data point is the average of three samples, with standard deviation shown by *error bars*.

Luciferase Protection Assays—Protection of firefly luciferase (luc) from thermal aggregation by sHSPs was assayed basically as described in Lee and Vierling (25). Heating reactions were prepared in 25 mM HEPES, pH 7.5, 15 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol (D buffer) to a final volume of 50 μl. Reactions had 24 or 96 μM sHSP and 1 μM luc. Samples were heated at 42 °C for 7.5 min, cooled on ice, and centrifuged at 16,000 × *g* for 20 min at 4 °C. Equal volumes of remaining soluble protein were run on a 14% SDS-PAGE and compared with unheated luc.

The ability of sHSPs to maintain luc in a re-foldable state was assayed in D buffer. Samples were heated at 42 °C, 7.5 min, then cooled on ice. Reactions were diluted into refolding buffer (60% rabbit reticulocyte lysate (Green Hectares, Oregon, WI) in D buffer with 2 mM ATP added). All samples were diluted to a final concentration of 30 nM sHSP in the refolding step, independent of the concentration during heat inactivation. To achieve this, samples were first diluted to 1.2 μM sHSP in D buffer with 6% reticulocyte lysate to protect the luc activity. In the absence of ATP, this mixture does not promote refolding. In the refolding reaction, heated samples were incubated at 31 °C for up to 2 h. Luciferase activity, relative to activity before heating, was determined by adding 2.5 μl of reaction to 50 μl of luc assay system (Promega) and measuring in a luminometer.

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 $MgSO_4$ and 44 °C heat stress was determined to best demonstrate sHSP-dependent survival. Fig. 1*A* 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 $MgSO_4$. In the absence of heat stress, there is no loss of viability by either strain on $MgSO_4$. When heated for 8 h at 44 °C on $MgSO_4$, 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 protein (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 *Pisum sativum* HSP18.1, was found to destabilize the sHSP oligomer *in vitro*.[4]

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. 2*A*, mutations of Leu-66 have varied effects. L66T has little effect on thermotolerance, whereas L66E and L66K are so deleterious that cells expressing these mutants 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. 2*B*). 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. Selec-

[4] D. S. Kim and E. Vierling, unpublished data.

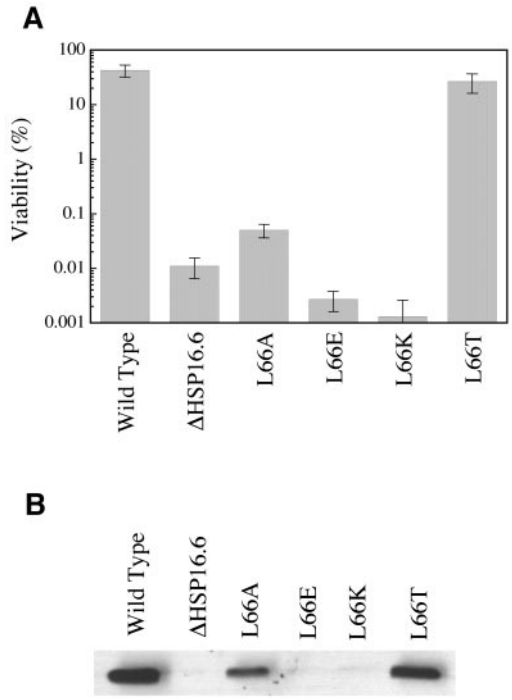

FIG. 2. **Heat stress sensitivity of strains with mutations of Leu-66 in HSP16.6.** *A*, viability of strains containing point mutants of Leu-66 in *hsp16.6* compared with wild-type HSP16.6 and ΔHSP16.6 strains (all in Δ*clpB1* background) after 8 h at 44 °C, as described in Fig. 1. Each *bar* represents 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).

tion for sHSP function was attempted with multiple cycles of heat shock and recovery. However, it became apparent that cells can become resistant to heat stress, even in the absence of an sHSP. After as few as two rounds of heat stress, a population of *hsp16.6* deletion cells became nearly as resistant to heat stress as +HSP16.6 and stayed resistant for many generations without further selection. Resistance also occurs in the ΔHSP16.6/ΔClpB1 strain. Based on the high frequency at which this occurs, it appears that sHSP-independent thermotolerance can be achieved by many different mechanisms, but this has not been pursued. As a result of this observation, only a single round of heat shock has been used to select for sHSP function.

The severe reduction of thermotolerance of cells carrying *hsp16.6 L66K* made this mutation appear to be an excellent tool to isolate suppressors that would restore sHSP function *in vivo*. 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* Δ*hsp16.6*/Δ*clpB1* 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 thermotolerance 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 (GCG to either CTG or TTG). Ala-66 to Thr (ACG), which can substitute for Leu-66 (Fig. 2*A*), was recovered.

The ability of the suppressors to restore thermotolerance is shown in Fig. 3*A*. 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. 3*B* 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. Thermotolerance 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 Thermotolerance—The suppressors of L66A have the potential to impair sHSP function in the absence of the L66A mutation. To test this, the thermotolerance 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 thermotolerance assay is not sensitive enough to measure. Reasoning that small defects of the suppressors might be additive in a double mutant, N40Y/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 thermotolerance. 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. 4*B*) 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. 4*A*, *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. 4*A*). 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
Suppressors of L66A

Suppressor mutant(s)	Independent isolates (no.)	Increase in hydrophobicity[a]	Thermotolerance of L66A with suppressor[b]	Thermotolerance of suppressor alone[b]
N40Y	3	6.5	+++	+++
T76I	1	7.5	++	+++
D80V	1	12.8	++	+++
D80H	1	4.1	++	ND[c]
D80N	1	2.1	++	ND
V108L	1	0.9	+++	+++
V133A	2	−2.2	+	+++
P8L/K137E	1	ND	++	+++
P8L		ND	+	+++
K137E		−1.3	+	+++

[a] As calculated by Radzicka and Wolfenden (38) for side-chain analogs, in kcal/mol. The hydrophobicity of proline was not determined.
[b] Scale is "+++" = wild-type thermotolerance, "−" = L66A.
[c] ND, not determined.

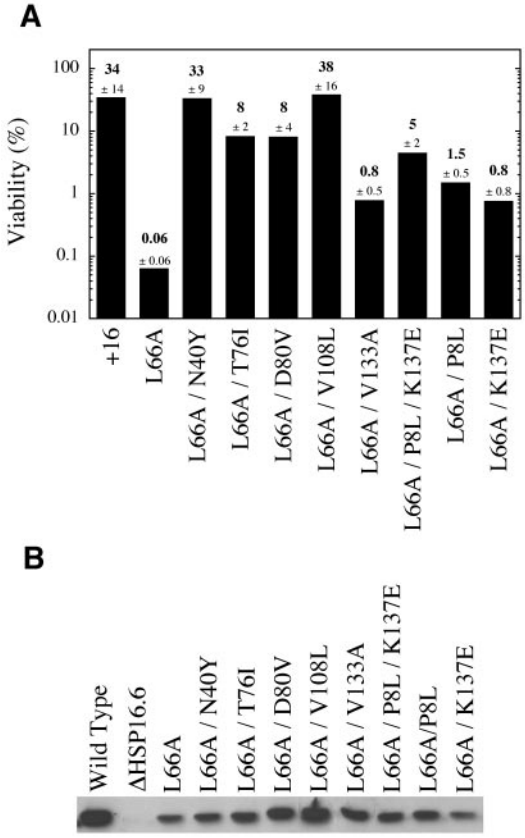

FIG. 3. **Suppression of thermotolerance defects of L66A by second-site mutations.** *A*, viability of strains containing suppressors of L66A compared with wild-type HSP16.6 and L66A (all in Δ*clpB1* background) after 8 h at 44 °C, as described in Fig. 1. Each *bar* represents the average of at least three samples. Average values and standard deviation are given *above bars*. *B*, accumulation of HSP16.6 in suppressor strains was determined by Western blot of lysates of cells treated at 42 °C for 2 h, as in Fig. 2.

V108L more than wild type, but still significantly less than L66A.

The single-mutant proteins D80V and V108L form very stable oligomers. Fig. 4*A* shows that, after being heated and cooled, the oligomerization of V108L is similar to wild type, and D80V is at least slightly more stable. To examine this more carefully, SEC was performed at an elevated temperature, and low concentration. At 38 °C and 6 μM, the wild-type HSP16.6 oligomer is the least stable of the three proteins, whereas the D80V oligomer is the most stable (Fig. 5). Thus, the mutations selected as suppressors of L66A create oligomers that are abnormally heat-stable.

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. 6*A*). 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. 6*B*). 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. 6*B* (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 reactivation 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.

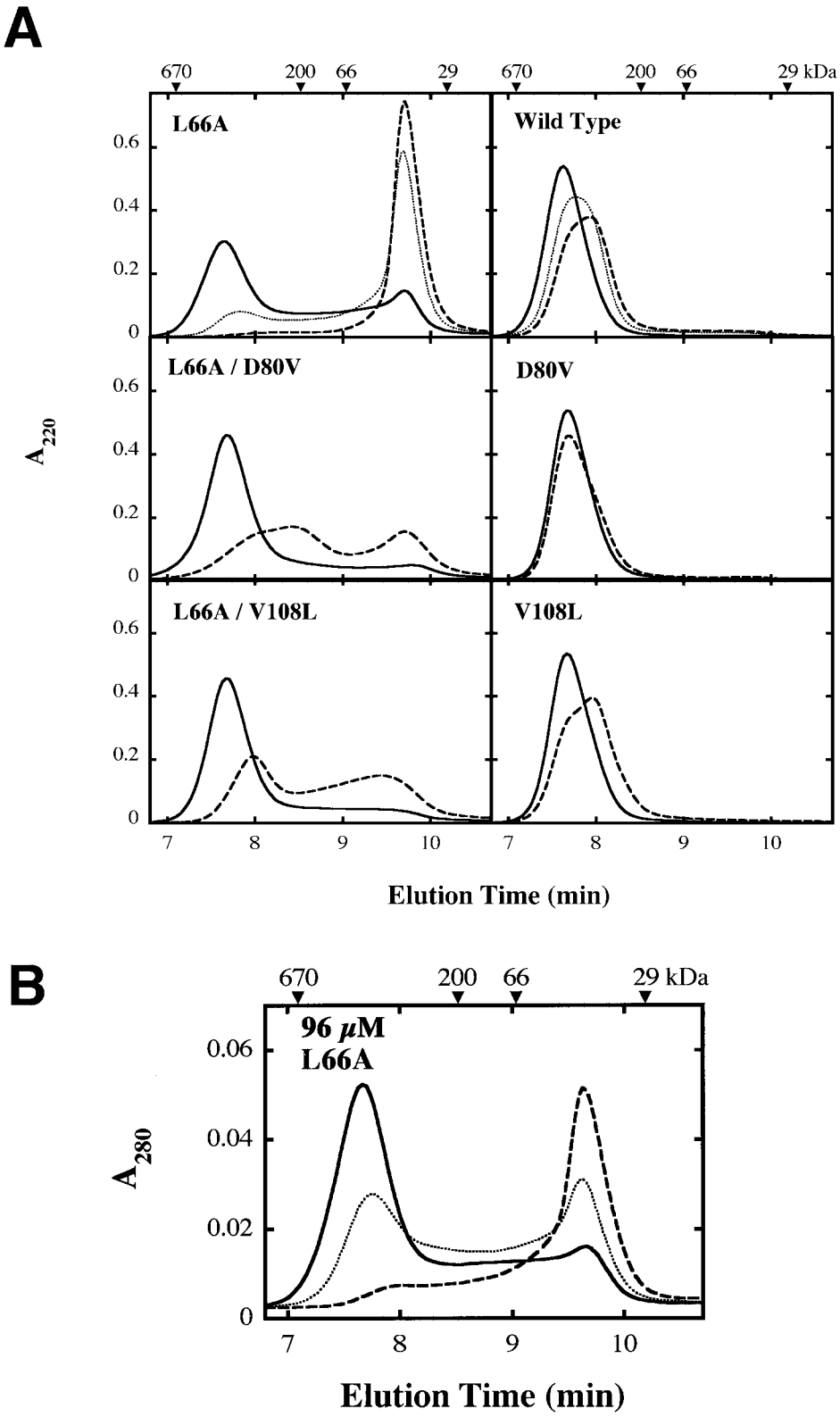

FIG. 4. **Oligomeric instability of L66A is repaired by suppressors.** SEC was performed at room temperature as described under "Experimental Procedures." *A*, 24 μM sHSP (detected at 220 nm). *B*, 96 μM sHSP (detected at 280 nm). Samples were kept at 4 °C (*solid line*), heated at 42 °C for 7.5 min and cooled at 4 °C for 20 min (*dashed line*), or heated as above and allowed to recover at 4 °C for 24 h before being injected onto column (*thin dotted line*). The peak heights between *A* and *B* are not directly comparable because absorbance in *B* was measured at 280 nm instead of the 220 nm used in *A*, to avoid saturating the detector. Elution times of protein standards are shown with *arrowheads*.

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. 7*B*). Lu-

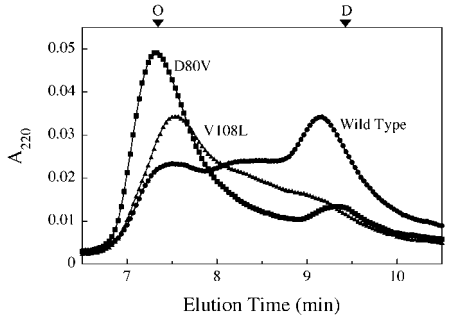

FIG. 5. **Oligomers of D80V and V108L are more stable than wild-type HSP16.6 at 38 °C.** SEC was performed at 38 °C. 6 μM wild type (*circles*), D80V (*squares*), or V108L (*triangles*) were injected onto column after being heated at 38 °C for at least 20 min. *Arrowheads* show the elution time of the oligomer (*O*) and of the suboligomeric species made by L66A (*D*).

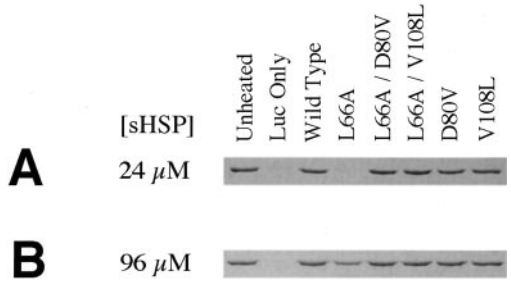

FIG. 6. **Protection of luc from aggregation by HSP16.6.** Luciferase was heated at 42 °C for 7.5 min in the absence or presence of HSP16.6 before being centrifuged. Equal volumes of the soluble fraction were run on a 14% SDS-PAGE gel, and Coomassie-stained. *A*, 1 μM luc with 24 μM sHSP; *B*, 1 μM luc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

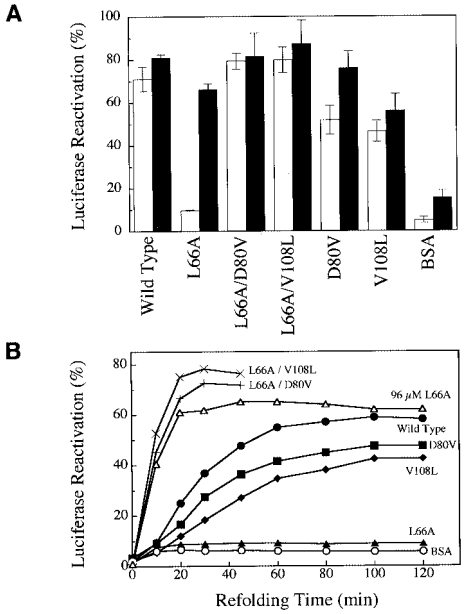

FIG. 7. **Reactivation of sHSP-protected luc.** 1 μM luc was heated at 42 °C 7.5 min in the presence of HSP16.6 and then diluted into refolding buffer, to a final sHSP concentration of 30 nM. Samples were assayed for luc activity at selected times and compared with activity before heating. *A*, maximum luc reactivation after protection by 24 μM (*light bars*) or 96 μM (*dark bars*) sHSP. BSA control contained an equivalent weight of protein (0.4 and 1.6 mg/ml). Data are the average of three experiments; *error bars* show standard deviation. *B*, time course of luc refolding, from a representative experiment, after being incubated with 24 μM sHSP wild-type (*filled circles*), L66A (*filled triangles*) L66A/D80V (*plus signs*), L66A/V108L (*crosses*), D80V (*squares*), V108L (*diamonds*); with 96 μM L66A (*open triangles*); or with 0.4 mg/ml BSA (*open circles*).

ciferase protected with wild type is less than half refolded at 20 min, whereas the $t_{1/2}$ of the double mutants are less than 10 min. This may be because of the effect of the L66A mutation. The 96 μM L66A reaction also has a $t_{1/2}$ of < 10 min. In contrast, the more strongly oligomerized mutants, D80V and V108L, allow much slower rates of luc refolding ($t_{1/2} \sim 30-40$ min). Although the mechanism by which luc moves from the sHSP to the ATP-dependent chaperones in reticulocyte lysate is not known, these data suggest that the rate of substrate refolding is limited by the sHSP and may be dependent on sHSP de-oligomerization.

DISCUSSION

The majority of what we know about sHSPs comes from *in vitro* studies with purified components. This previous work has clearly demonstrated that sHSPs recognize misfolded proteins and maintain them in a soluble but inactive state, but has not addressed the key question of whether this activity is important to the *in vivo* function of sHSPs. We have characterized the effects of mutations that alter sHSP oligomeric stability both *in vivo* and *in vitro*. By combining genetics with biochemistry, we have shown that a mutant that cannot suppress aggregation of a model substrate is also defective *in vivo*, thus providing direct support for the chaperone model of sHSP function.

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 vivo* 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 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-competent 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

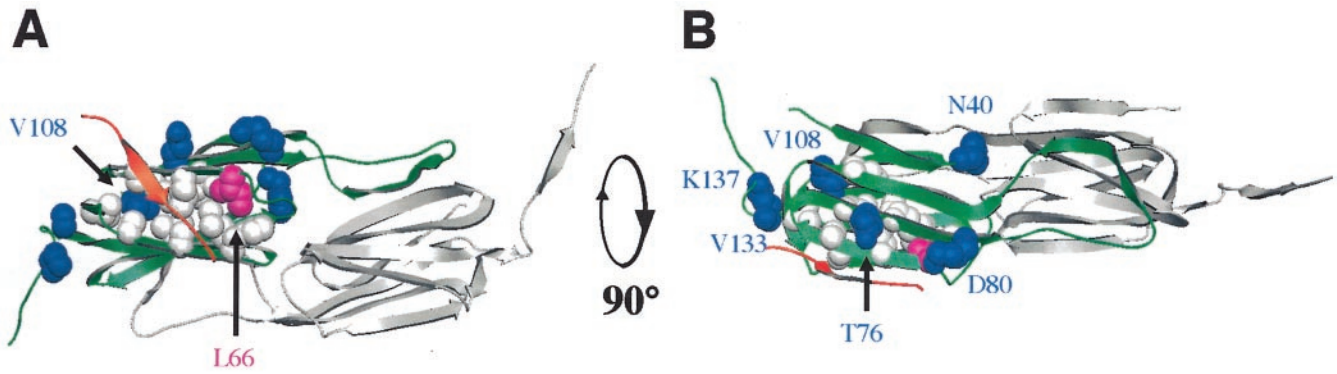

FIG. 8. **Predicted location of mutants on sHSP dimer structure.** The ribbon structure of a dimer (*green* and *gray*) of MjHSP16.5 (8) with the C-terminal arm from another dimer shown in *orange*. *A*, view of outer surface of oligomer, so that inside of oligomer is within the page. Hydrophobic patch 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).

TABLE II
Summary of biochemical data

Protein	Thermotolerance[a]	Oligomer stability[b]	Luciferase solubility[b,c]	Luciferase reactivation	
				Yield[c]	$\sim t_{1/2}$[d]
				%	*min*
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] Activities of mutants relative to wild-type HSP16.6 = "+++."
[c] Based on experiments performed with 1 μM luc heated with 24 μM sHSP.
[d] Estimated from three experiments, with an error of ± 2 min. 1 μM luc heated with 96 μM L66A or 24 μM other sHSPs.

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 a mechanism that does not require normal assembly of a complex, such as noncooperative binding of substrate by independent dimers. In this way a high concentration of the mutant may be able to protect a small amount of luc. Similar results were obtained by Feil *et al.* (35), who made a dimeric fragment of αB-crystallin. This dimer protected alcohol dehydrogenase from aggregation, but needed to be 25 times more concentrated than the wild type αB-crystallin. Thus, substrate protection by nonoligomeric sHSPs can be very inefficient compared with sHSPs that are capable of oligomerizing.

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. 8*A*). 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. 8*B*). 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. 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

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 reactivation assay (Table II). 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 reactivation 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 II). 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 sHSP 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 IbpB (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.

Acknowledgments—We gratefully acknowledge H. Kosaka and H. Fukuzawa, of Kyoto University, for generously communicating unpublished results and providing plasmids and *Synechocystis* strains. We thank J. Little and B. Patterson for helpful discussions, and K. Friedrich, A. Hausrath, and J. Little for critical reading of the manuscript.

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

REFERENCES

1. Horwitz, J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 10449–10453
2. Van Montfort, R., Slingsby, C., and Vierling, E. (2002) *Adv. Protein Chem.* **59,** 105–156
3. Haslbeck, M., Walke, S., Stromer, T., Ehrnsperger, M., White, H. E., Chen, S., Saibil, H. R., and Buchner, J. (1999) *EMBO J.* **18,** 6744–6751
4. van Boekel, M. A., de Lange, F., de Grip, W. J., and de Jong, W. W. (1999) *Biochim. Biophys. Acta* **1434,** 114–123
5. van Montfort, R. L. M., Basha, E., Friedrich, K. L., Slingsby, C., and Vierling, E. (2001) *Nat. Struct. Biol.* **8,** 1025–1030
6. Ehrnsperger, M., Graber, S., Gaestel, M., and Buchner, J. (1997) *EMBO J.* **16,** 221–229
7. Lee, G. J., Roseman, A. M., Saibil, H. R., and Vierling, E. (1997) *EMBO J.* **16,** 659–671
8. Kim, K. K., Kim, R., and Kim, S. H. (1998) *Nature* **394,** 595–599
9. de Jong, W. W., Caspers, G. J., and Leunissen, J. A. (1998) *Int. J. Biol. Macromol.* **22,** 151–162
10. Lavoie, J. N., Gingras-Breton, G., Tanguay, R. M., and Landry, J. (1993) *J. Biol. Chem.* **268,** 3420–3429
11. Soto, A., Allona, I., Collada, C., Guevara, M. A., Casado, R., Rodriguez-Cerezo, E., Aragoncillo, C., and Gomez, L. (1999) *Plant Physiol.* **120,** 521–528
12. Petko, L., and Lindquist, S. (1986) *Cell* **45,** 885–894
13. Thomas, J. G., and Baneyx, F. (1998) *J. Bacteriol.* **180,** 5165–5172
14. Plesofsky-Vig, N., and Brambl, R. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 5032–5036
15. Lee, S., Owen, H. A., Prochaska, D. J., and Barnum, S. R. (2000) *Curr. Microbiol.* **40,** 283–287
16. Kuczynska-Wisnik, D., Kcdzierska, S., Matuszewska, E., Lund, P., Taylor, A., Lipinska, B., and Laskowska, E. (2002) *Microbiology* **148,** 1757–1765
17. Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M., and Tabata, S. (1996) *DNA Res.* **3,** 109–136
18. Vermaas, W. F. (1998) *Methods Enzymol.* **297,** 293–310
19. Elhai, J., and Wolk, C. P. (1988) *Gene* (*Amst.*) **68,** 119–138
20. Clos, J., and Brandau, S. (1994) *Protein Exp. Purif.* **5,** 133–137
21. Williams, J. G. K. (1988) *Methods Enzymol.* **167,** 766–778
22. Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., and Stanier, R. Y. (1979) *J. Gen. Microbiol.* **111,** 1–61
23. Leung, D. W., Chen, E., and Goeddel, D. V. (1989) *Technique* **1,** 11–15
24. Ghosh, S., Gepstein, S., Heikkila, J. J., and Dumbroff, E. B. (1988) *Anal. Biochem.* **169,** 227–233
25. Lee, G. J., and Vierling, E. (1998) *Methods Enzymol.* **290,** 350–365
26. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) *Protein Sci.* **4,** 2411–2423
27. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
28. Parsell, D. A., Kowal, A. S., Singer, M. A., and Lindquist, S. (1994) *Nature* **372,** 475–478
29. Glover, J. R., and Lindquist, S. (1998) *Cell* **94,** 73–82
30. Zolkiewski, M. (1999) *J. Biol. Chem.* **274,** 28083–28086
31. Eriksson, M. J., and Clarke, A. K. (1996) *J. Bacteriol.* **178,** 4839–4846
32. Eriksson, M. J., Schelin, J., Miskiewicz, E., and Clarke, A. K. (2001) *J. Bacteriol.* **183,** 7392–7396
33. Török, Z., Goloubinoff, P., Horvath, I., Tsvetkova, N. M., Glatz, A., Balogh, G., Varvasovszki, V., Los, D. A., Vierling, E., Crowe, J. H., and Vigh, L. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 3098–3103
34. Lee, G. J., and Vierling, E. (2000) *Plant Physiol.* **122,** 189–198
35. Feil, I. K., Malfois, M., Hendle, J., van der Zandt, H., and Svergun, D. I. (2001) *J. Biol. Chem.* **276,** 12024–12029
36. Veinger, L., Diamant, S., Buchner, J., and Goloubinoff, P. (1998) *J. Biol. Chem.* **273,** 11032–11037
37. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis* **18,** 2714–2723
38. Radzicka, A., and Wolfenden, R. (1988) *Biochemistry* **27,** 1664–1670