The Identity of Proteins Associated with a Small Heat Shock Protein during Heat Stress in Vivo Indicates That These Chaperones Protect a Wide Range of Cellular Functions*

Eman Basha‡‡§§, Garrett J. Lee¶†, Linda A. Breci, Andrew C. Hausrath‡, Nicole R. Buan‡**†, Kim C. Giese‡, and Elizabeth Vierling‡‡§§

From the Departments of ©Biochemistry and Molecular Biophysics, ‡‡Molecular and Cellular Biology, and †Chemistry, University of Arizona, Tucson, Arizona 85721-0106

The small heat shock proteins (sHSPs) are a ubiquitous class of ATP-independent chaperones believed to prevent irreversible protein aggregation and to facilitate subsequent protein renaturation in cooperation with ATP-dependent chaperones. Although sHSP chaperone activity has been studied extensively in vitro, understanding the mechanism of sHSP function requires identification of proteins that are sHSP substrates in vivo. We have used both immunoprecipitation and affinity chromatography to recover 42 proteins that specifically interact with Synechocystis Hsp16.6 in vivo during heat treatment. These proteins can all be released from Hsp16.6 by the ATP-dependent activity of DnaK and co-chaperones and are heat-labile. Thirteen of the putative substrate proteins were identified by mass spectrometry and reveal the potential for sHSPs to protect cellular functions as diverse as transcription, translation, cell signaling, and secondary metabolism. One of the putative substrates, serine esterase, was purified and tested directly for interaction with purified Hsp16.6. Hsp16.6 effectively formed soluble complexes with serine esterase in a heat-dependent fashion, thereby preventing formation of insoluble serine esterase aggregates. These data offer critical insights into the characteristics of native sHSP substrates and extend and provide in vivo support for the chaperone model of sHSP function.

The small heat shock proteins (sHSPs) and the structurally related vertebrate eye lens α-crystallins are a family of virtually ubiquitous stress proteins (1, 2). Their role in cellular stress extends from protection against high temperature and oxidative stress to a potentially important function in a variety of protein-folding diseases and aging (3, 4). The sHSPs are defined by a conserved C-terminal domain of ~90 amino acids (the α-crystallin domain), which is flanked by a variable length N-terminal arm and a more conserved C-terminal extension (1, 5). These small proteins (16–42 kDa monomers) assemble into oligomeric structures of 9 to >32 subunits depending on the sHSP (6, 7). sHSPs are very efficient at binding denatured proteins, and current models propose that they function as ATP-independent molecular chaperones to prevent irreversible protein aggregation and insolubilization (2). sHSP-bound proteins can be reactivated by the ATP-dependent chaperone activity of DnaK/Hsp70, with the help of ClpB or GroEL in some instances (8–10). Thus, sHSPs are proposed to be a critical component of the cellular chaperone network that becomes particularly important under conditions of severe stress involving protein aggregation.

Although the ability of sHSPs to interact with denatured model substrates in vitro has been studied extensively, the characteristics of cellular sHSP substrates remain poorly defined. Understanding the mechanism of sHSP chaperone action, as well as determining how these proteins may act to protect cells during stress, requires identification of proteins that interact with sHSPs in vivo, either as partners or substrates. Co-immunoprecipitation, two-hybrid analysis, and other techniques have been used to identify potential sHSP-interacting proteins in several systems (2). Such studies have identified a diversity of proteins suggested to associate with sHSPs. These include a chondroitin sulfate proteoglycan (11), a ubiquitin-conjugating enzyme (12), a transacting DNA-binding protein involved in the response to glucocorticoids (13), a proteasomal subunit (14), an F-box protein (15), myotonic dystrophy protein kinase (16), and eukaryotic initiation factor 4G (17). In vertebrates, sHSPs are proposed to interact directly with actin and intermediate filament proteins, potentially altering the filament assembly/disassembly equilibrium of these cytoskeletal components (18–22). The idea that sHSPs might have multiple substrates is supported by observation in plants, yeast, and mammals, where there is evidence that many different proteins associate with sHSPs during heat stress (23–25). However, none of these latter studies identified any of the interacting proteins.

We are investigating sHSP function using a model genetic organism, the cyanobacterium Synechocystis sp. PCC 6803 (26). Critical studies of sHSP function have been hampered by the lack of any of or easily assayed phenotypes associated with sHSP deletions in Saccharomyces cerevisiae or Escherichia coli (27, 28). The Synechocystis genome encodes a single sHSP, Hsp16.6, deletion of which leads to a conditional lethal phenotype after high temperature treatment (26). Like other sHSPs,
Hsp16.6 is oligomeric (–20 subunits) and in vitro can facilitate refolding of bound denatured proteins in conjunction with the DnaK/Hsp70 chaperone system and, under some conditions, most likely also with the aid of ClpB (9, 26). We have used both immunoprecipitation and affinity chromatography to show that at least 42 proteins interact with Hsp16.6 in heat-stressed cells, 13 of which were subsequently identified by mass spectrometry. Hsp16.6 proteins fit stringent criteria for being shHSP substrates, including specific interaction under heat stress conditions, ability to be released from Hsp16.6 by the DnaK system plus ATP, and heat lability. We also show that, in a purified system, one of these putative substrate proteins forms complexes with Hsp16.6 at high temperature. The proteins identified show no commonality in sequence or structure that could be a signature for an shHSP substrate. The range of functions of these putative substrate proteins provides evidence that shHSPs are important for protection of multiple cell proteins and a wide variety of cellular activities, including translation, transcription, cell signaling, and secondary metabolism.

EXPERIMENTAL PROCEDURES

Synechocystis Strains and Growth Conditions—Isogenic wild-type and hsp16.6 deletion (Δhsp16.6) strains expressing a spectinomycin resistance gene (aadA) were created as described previously (26, 51), and all experiments specifying the wild type in this work were performed with this spectinomycin-resistant wild-type strain. A strain was also generated in which the wild-type with this spectinomycin-resistant wild-type strain. A strain was also generated to include an 8-amino acid C-terminal Strep-tag II affinity tag (WSHPQFEK) (54, 55). Cells were propagated in medium 10-fold, spotted onto BG-11 agar plates, and grown under light estimating cell viability, cells were serially diluted six times in BG-11 supplemented with 5 mM glucose in a shaking culture under lights (60 rpm).

Streptactin Affinity Chromatography—The strain in which the hsp16.6-strep gene was substituted for the wild-type gene was grown in a 50-ml culture to A730 = 0.15 and subjected to heat stress or control conditions as described above for the radioactive labeling experiments. The cells were separated in lysis buffer minus apyrase, but with 1 mM EGTA and 50 mM NaCl (pH 7.5). The dead cells were harvested by centrifugation. Samples were analyzed by SDS-PAGE and transferred to nitrocellulose membranes. Radiolabeled proteins were co-immunoprecipitated with Hsp16.6-strep (protein A 50 µl, 1:1000; Sigma) plus 30 µl of streptactin resin (Sigma) at 4 °C for 2 h. The resin was washed, and bound proteins were eluted and analyzed as described for immunoprecipitation.

Gel Electrophoresis—SDS-PAGE was performed according to standard protocols, using 10–17% acrylamide gradient gels to maximize resolution. For two-dimensional gels, immunoprecipitated proteins were extracted with 1× SDS sample buffer (2% SDS, 150 mM Tris (pH 8.8), 160 mM dithiothreitol, 5 mM e-aminoacrylic acid, and 1 mM benzamidine) and then precipitated with 5 × 0.1 M ammonium acetate in methanol (v/v) overnight at –20 °C. The precipitates were centrifuged for 15 min at 16,250 × g and washed twice with ammonium acetate in methanol and then twice with ice-cold acetone. The air-dried pellets were dissolved in rehydration buffer (9.0 M urea, 2% CHAPS, 2% IPG buffer pH 3–10, 0.5% 2-hydroxypropyl-β-cyclodextrin). The pH 3–10 NL first dimension strips (13 cm; Amersham Biotech) were then rehydrated overnight at room temperature using 250 µl of sample in rehydration buffer. Focusing was carried out for 2 h at 150 V, 300 V, 400 V, 500 V, 600 V, 750 V, 800 V, 1000 V, and 1500 V. The second dimension was run on SDS-11–17% polyacrylamide gels for 30 min at 15 mA and then for 7 h at 25 mA. Silver staining was performed according to Rabilloud et al. (61).

Identification of Proteins by Mass Spectrometry—The total insoluble protein fraction prepared as described for immunoprecipitation samples (from 50 ml of heat-stressed cells) was separated by two-dimensional gel electrophoresis and stained with Coomassie Blue. Polypeptides identified as heat-associated with Hsp16.6 and released by DnaK, DnaJ, and GrpE were excised from the gel and subjected to trypsin digestion according to Shevchenko et al. (62). Digested samples were cleaned and concentrated on ZipTip (Millipore Corp., Bedford, MA) and mixed 1:5 in 0.1% c-cyano-4-hydroxycinnamic acid. Mass analysis was performed on a Bruker Reflex-III MALDI-TOF instrument (Bruker-Daltonics, Inc.) using internal standards that improved mass accuracy over the mass range of m/z 904–2465, with 10–30 ppm error observed.

Peptide mass matching for protein identification was performed using the Protein Prospector program at University of California, San Francisco. The identification was performed with the NCBi Database. The search was done with no restriction of molecular mass, pl, or organism. The following criteria were used for identification: the error was kept under 50 ppm, and identified peptides covered at least 19% of the identified protein.

Hsp16.6 is a 16-kDa protein that is expressed in Synechocystis sp. PCC 6803 (open reading frame sll1514), or DnaK and Hsp70, in conjunction with the DnaK/Hsp70 chaperone system and, under some conditions, most likely also with the aid of ClpB. The proteins identified show no commonality in sequence or structure that could be a signature for an shHSP substrate. The range of functions of these putative substrate proteins provides evidence that shHSPs are important for protection of multiple cell proteins and a wide variety of cellular activities, including translation, transcription, cell signaling, and secondary metabolism.

**Experimental Procedures**

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Cloning and Purification of Soluble Serine Esterase—The coding sequence for serine esterase (open reading frame sll1284) was amplified from Synechocystis genomic DNA by PCR using primers 5'-TGTACAT-ATGGGCTCTACATTTTTCGGCG-3' and 5'-TCGAGAATTCTCTCAACTCA-AAAAGACTTTAAG-3'. The resulting 632-bp fragment was cloned into expression vector pET28b (Novagen, Madison, WI) with NdeI and EcoRI to incorporate an N-terminal His tag and was confirmed by sequence analysis. Protein expression was carried out in BL21(DE3) cells grown in LB broth at 37 °C to an OD with an inducer and induced for 4 h with 1 mM isopropyl-β-D-thiogalactopyranoside. Cells from 10-ml cultures were collected by centrifugation and resuspended in 300 ml of 50 mM Tris-HCl (pH 8.0) and 400 mM NaCl (Buffer A) and lysed with a French press, and insoluble material was removed by centrifugation. The soluble serine esterase, which was only a small percentage of the total expressed protein, was retained on a Talon metal affinity column (Clontech), washed with Buffer A plus 10 mM imidazole, and then eluted in Buffer A plus 500 mM imidazole. The eluate was precipitated by addition of ammonium sulfate to 70% saturation followed by centrifugation, resuspended in a minimum volume of Buffer A, and applied to a Sephacryl S-100 HR column equilibrated in the same buffer. Fractions containing pure serine esterase were selected after SDS-PAGE and mass spectrometry of the purified enzyme gave a mass of 24,233.9 Da, consistent with the theoretical mass without the N-terminal 14,000 rpm in an Eppendorf tabletop centrifuge. For supernatant samples, 70 μl of supernatant was removed from each mixture. The pellet was then washed with 1 ml of buffer; after centrifugation, 960 μl of wash was removed, and the remainder of the sample comprised the pellet samples.

Size-exclusion chromatography was used to confirm a physical association of serine esterase and Hsp16.6. Samples (100 μl) with 10 μM serine esterase and/or 20 μM Hsp16.6 were prepared as described above, held at room temperature or heated for 20 or 60 min at 46 °C, and centrifuged as described above. Supernatant (70 μl) was injected onto a Bio-Sil SEC400 column (Bio-Rad) on a Rainin Instrument high pressure liquid chromatography system. The column was equilibrated in 50 mM Tris (pH 7.8) and 75 mM NaCl and run at a flow rate of 1 ml/min at 25 °C while absorbance was recorded at 220 nm.

RESULTS

Induction of Thermotolerance and Accumulation of Hsp16.6—To establish conditions under which to examine Hsp16.6 interactions with other proteins in vivo, we first characterized the growth of Synechocystis isogenic wild-type and Δhsp16.6 strains and determined the levels of Hsp16.6 after heat treatment in liquid culture. The isogenic wild-type and deletion strains had the same growth rate at the 30 °C optimum growth temperature. Although maximum levels of Hsp16.6 accumulated after 60-120 min at 42 °C (data not shown), this treatment did not affect cell viability in wild-type Hsp16.6 or the Δhsp16.6 mutant (data not shown). These results are consistent with previous studies of Synechocystis wild-type Hsp16.6 and an Hsp16.6 insertional mutant (29, 30). A 42 °C treatment for 120 min was therefore chosen as a pretreatment to allow accumulation of Hsp16.6 for experiments aimed at examining interactions of the sHSP with other proteins.

Temperature treatments that revealed phenotypic differences between the wild-type and Δhsp16.6 strains in liquid culture were determined, and the solubility of Hsp16.6 under the same conditions was examined. The effect of a 120-min pretreatment at 42 °C on subsequent survival of a more severe 46 °C treatment was investigated (Fig. 1A). A 46 °C treatment (0–90 min) was applied after the pretreatment (approximately one cell generation), during which time the cells were grown at 30 °C to provide time for uniform protein labeling with isotopic amino acids in subsequent experiments. Strain viabilities from a representative experiment are shown in Fig. 1B. A difference in viability between the wild-type and deletion strains became apparent after 40 min at 46 °C; and after 90 min, the wild-type strain had approximately an order of magnitude greater viability than the deletion strain. By 120 min, the difference in viability typically increased to >3 orders of magnitude (data not shown). The same ~10-fold difference in viability was seen when cells were placed directly at 46 °C after the 42 °C treatment or when cells were allowed to recover for 5, 6.5, or 12 h before the 46 °C treatment. Thus, Hsp16.6 makes a significant contribution to the acquisition of cellular thermotolerance under these conditions.

The level and solubility of Hsp16.6 over the course of the experiment were then examined. Total cell proteins were extracted in immunoprecipitation buffer, and soluble and insoluble fractions were separated by centrifugation. Hsp16.6 was not detected in the wild-type cells grown at 30 °C, but significant levels accumulated in a soluble form after the 42 °C pre-
treatment (Fig. 1C). When the cells were returned to their normal 30 °C growth temperature, Hsp16.6 levels remained fairly constant for up to 24 h. Maximum accumulation of Hsp16.6 was determined to represent 0.5% of the total cell protein by Western analysis of purified Hsp16.6 compared with protein from heat-stressed cells (data not shown). If cells were then restressed at the higher temperature of 46 °C for 20 min, there was no significant increase in the level of Hsp16.6, and about one-half of the pre-existing Hsp16.6 remained soluble, whereas the rest was recovered in the pellet fraction. Longer treatment at 46 °C did not significantly increase the fraction of insoluble Hsp16.6 (data not shown). These stress conditions, which allowed accumulation of soluble Hsp16.6 and under which the presence of Hsp16.6 led to significant protection of viability, were chosen for further study of Hsp16.6 interaction with cell proteins.

Numerous Synechocystis Proteins Co-immunoprecipitate with Hsp16.6 in Cells Treated at High Temperature—We next sought to determine whether Hsp16.6 shows a temperature-dependent interaction with any endogenous Synechocystis proteins, as would be predicted by the chaperone model for sHSP activity. As described above (Fig. 1A), exponentially growing wild-type cells were heat-stressed for 120 min at 42 °C to allow accumulation of Hsp16.6, returned to 30 °C, and grown in the presence of [35S]Met for 11 h. Radiolabeled cell cultures were divided in half, and one aliquot was maintained at 30 °C (conditioned cells), whereas the other aliquot was restressed at 46 °C for 20 min (heat-stressed cells). The short 46 °C treatment was chosen to minimize effects due to death of a large number of cells because both wild-type and Δhsp16.6 strains were >85% viable at this time point (Fig. 1B). However, we assume that interactions initiated in the first 20 min of stress are critical to the difference in survival between the wild-type and Δhsp16.6 strains that develops over time at 46 °C. This 46 °C treatment also did not lead to significant differences in the pattern of labeled proteins in the conditioned and heat-stressed cell samples, such that immunoprecipitation experiments were being performed with equivalently labeled cell proteins (Fig. 2A, lanes 8 and 9).

Cells were lysed and processed for immunoprecipitation with Hsp16.6 antiserum or preimmune serum. Hsp16.6 was effectively immunoprecipitated from wild-type cells both before and after the short 46 °C heat treatment, but comparison of equal quantities of immunoprecipitate from the prestressed and heat-stressed samples revealed that many more proteins specifically coprecipitated with Hsp16.6 after the heat stress (Fig. 2A, lanes 4 and 5). The majority of proteins coprecipitating with Hsp16.6 in the prestressed sample can be considered nonspecific after comparison with the Δhsp16.6 and preimmune controls (Fig. 2A, lanes 1 and 2 and lanes 6 and 7). In an additional sample, cells of the deletion strain that had received the 42 °C pretreatment and 46 °C treatment were lysed in the presence of [35S] Met for 11 h. Radiolabeled phycocyanins migrating at 17 kDa were not found in the immunoprecipitates. Also, in the proteins associated with Hsp16.6, there was major enrichment of many proteins above ~60 kDa. The same spectrum of proteins associated with Hsp16.6 was also seen when cells were labeled for 5, 6.5, or 12 h...
at 30 °C prior to the 46 °C heat stress (data not shown). The polypeptides that showed a temperature-dependent interaction with Hsp16.6 represent potential substrates protected by Hsp16.6 chaperone activity.

To obtain a clearer picture of the number of proteins associated with Hsp16.6 during heat shock, immunoprecipitated proteins in samples equivalent to those in Fig. 2A (lanes 4 and 5) were analyzed by two-dimensional electrophoresis (Fig. 2B). Comparison of the heat-shocked immunoprecipitate with the control sample, as well as with preimmune and Δhsp16.6 samples (with or with added purified Hsp16.6) (data not shown), allowed us to identify 27 polypeptides that were reproducibly associated with Hsp16.6 during heat shock, immunoprecipitated proteins. These polypeptides range from 14 to 64 kDa with pI values from an estimated 4.5 to >7.5.

Because the two-dimensional separation poorly represents Hsp16.6-associated proteins above ~80 kDa, proteins in this size range were resolved on SDS-acrylamide gradient gels at a lower percentage (11–13%). Six abundant and nine minor polypeptides were identified in this size range (data not shown), bringing the total of easily identified Hsp16.6-associated proteins to 42.

**Endogenous Proteins Bound to Hsp16.6 Can Be Released by Addition of the DnaK Chaperone System and ATP**—The model for sHSP activity predicts that proteins bound to Hsp16.6 represent substrates that can be refolded by the DnaK molecular chaperone system (2). To test this prediction, Hsp16.6 immunoprecipitates prepared from cells treated at 46 °C as described above were incubated under protein refolding conditions in the presence of ATP and E. coli DnaK, DnaJ, and GrpE (Fig. 3A). Quantitation of counts/min released over time showed rapid release of Hsp16.6-bound radioactivity. Release of radioactivity was strictly dependent on ATP and the presence of all three components of the DnaK refolding system. The kinetics of release also compare favorably with refolding of model substrates protected by sHSPs (8, 31), and the reaction was essentially complete within 60 min. The ability of the E. coli system to work effectively with the *Synechocystis* sHSP is consistent with our previous observations (8, 9).

To confirm that the radioactivity released represented those polypeptides shown to interact specifically with Hsp16.6 at high temperature, bound and released fractions from the 60-min time point were analyzed by SDS-PAGE (Fig. 3B). It is very clear that a significant proportion of the specifically bound polypeptides were released from the immunoprecipitates in an ATP- and DnaK chaperone-dependent fashion (Fig. 3B, lane 6). In contrast and consistent with the quantitation results, only a few, nonspecific polypeptides were recovered in the released fraction when any component of the refolding system was left out of the reaction or when release was performed with immunoprecipitates from prestressed cells (Fig. 3B, lanes 8, 10, and 12 and lane 3, respectively).

To better determine the number of released proteins, the bound and released fractions from samples equivalent to those in Fig. 3B (lanes 5 and 6 and lanes 7 and 8) were separated by two-dimensional gel electrophoresis (Fig. 4). The same polypeptides found in Fig. 2B to be specific to the heat-shocked sample could all be identified as ATP-dependent for release, and a subset of these are indicated with arrows.

**Hsp16.6-associated Proteins Represent Proteins That Become Insoluble During Heat Stress**—It was expected that Hsp16.6-associated proteins would include proteins that are denatured and potentially become insoluble during heat stress. Under the conditions of our experiments, in samples prepared for immunoprecipitation from prestressed cells, 5% of the total cellular radioactivity was in the pellet fraction, whereas pellet samples from heat-shocked cells contained 10% of the total radioactivity. The relationship of Hsp16.6-associated proteins and specific proteins that become insoluble during heat stress was examined by two-dimensional electrophoresis. Essentially all of the proteins detected in the Hsp16.6 heat-shocked immunoprecipitate could be detected at some level in the radioactive insoluble cell fraction (data not shown). There were no heat-induced insoluble proteins that were not detected in the Hsp16.6 immunoprecipitate. However, Hsp16.6 was not associated with major insoluble proteins that are also insoluble under control conditions. These results indicate that Hsp16.6 can interact with many different heat-sensitive aggregating proteins, but does not bind to proteins that are detergent-insoluble in the absence of stress.

**Identification of Hsp16.6-associated Proteins**—As discussed above, all of the proteins specifically associated with Hsp16.6 also partitioned to some extent into the insoluble cell fraction. The insoluble protein fraction was also much less complex than the soluble protein fraction. Therefore, the insoluble protein fraction was used to identify Hsp16.6-associated proteins, leading to clearer isolation of individual polypeptide spots. Total insoluble proteins (from cells treated as described for the immunoprecipitation experiment; Fig. 2) were combined with radioactively labeled immunoprecipitates and separated by two-dimensional electrophoresis. Gels were stained with Coomassie Blue and autoradiographed, and the coincidence of radioactive and stained polypeptides was determined. Twenty-seven polypeptides corresponding to ones found to be bound specifically to Hsp16.6 and to be released by DnaK, DnaJ, and GrpE were easily detected in the total insoluble cell fraction. These polypeptides were excised from duplicate Coomassie Blue-stained gels, digested with trypsin, and processed for analysis by MALDI-TOF mass spectrometry.

From the 27 polypeptides identified on two-dimensional gels as interacting with Hsp16.6, 13 were clearly identified (Table 1). All of these proteins met the criteria that they were specifically associated with Hsp16.6 in the immunoprecipitations from heat-shocked cells, could be released from immunoprecipitates in the presence of the DnaK system and ATP, and also accumulated to some extent in the insoluble cell fraction during heat stress. The proteins participate in a diverse array of cellular functions, including translation, transcription, secondary metabolism, cell signaling, and other processes.

**The Same Putative Substrates Copurify with Affinity-tagged Hsp16.6**—We sought an independent method to confirm the results of the immunoprecipitation experiments and of the identification of Hsp16.6-associated proteins. A *Synechocystis* strain was constructed in which the hsp16.6 gene was engineered to include an 8-amino acid C-terminal affinity tag (Strep-tag II). This Hsp16.6-strep strain is fully isogenic to our wild-type strain, with the exception of the C-terminal tag on Hsp16.6. We have shown previously that the affinity tag does not significantly affect growth or viability under control or heat stress conditions compared with the wild-type strain (63). Wild-type Hsp16.6, Δhsp16.6, and Hsp16.6-strep cells were treated as described for the immunoprecipitation experiments (Fig. 1A) to obtain prestressed and 46 °C heat-stressed samples, and soluble cell extracts were processed by affinity chromatography to obtain Hsp16.6-associated proteins. As in the immunoprecipitation experiments, purified recombinant Hsp16.6-strep protein was added to a heat-stressed Δhsp16.6 cell sample prior to lysis to control for nonspecific interactions.

The stained gel of the affinity column eluates (Fig. 5) reveals a pattern of sHSP-associated proteins fully comparable with that observed in the radioactive labeling experiment in Fig. 2. Two-dimensional electrophoretic analysis of these same sam-
amples further confirmed that the radiolabeled Hsp16.6-associated proteins, identified by immunoprecipitation, were coincident with the stained proteins from this affinity experiment (data not shown). The ratio of specific sHSP-associated proteins to recovered Hsp16.6 can be estimated from the staining pattern as approximately an equal mass of substrate to sHSP. Note that the amount of added purified sHSP in Fig. 5 (fourth lane, Δ16/S) closely matches the cellular level, as predicted from our estimates of the in vivo accumulation of Hsp16.6 under these conditions (0.25% of the soluble protein). To confirm the identification of specific substrates, antibodies against Synechocystis ferredoxin-NADP⁺ reductase and heme oxygenase were used to analyze Western blots of the same samples. Heme oxygenase was detected only in samples from heat-stressed cells, and association did not occur with exogenously added sHSP. Ferredoxin-NADP⁺ reductase was also strongly enriched in the heat-stressed cell sample, although a slight increase above nonspecific interactions was seen in the pre-stressed sample prior to the 46 °C heat stress. These data indicate that the MALDI experiments correctly identified these proteins, indicating that these and other proteins in Table I are candidates for Hsp16.6 substrates.

Serine Esterase Is Protected from Heat-induced Insolubilization and Complexes with Hsp16.6 in Vitro—In accordance with
The ability of Hsp16.6 to protect serine esterase from insolubilization was then tested at different ratios of serine esterase to sHSP, holding the serine esterase concentration constant at 10 μM. When heated for 20 min at 46 °C, between 5 and 20 μM Hsp16.6 (monomer) was sufficient to maintain essentially all of the serine esterase in a soluble form (Fig. 6, fifth and seventh lanes). A 60-min incubation at 46 °C required somewhat higher levels of Hsp16.6 to afford complete protection, but protection was still complete at a ratio of 20 μM sHSP to 10 μM serine esterase (data not shown). The same mass of bovine serum albumin had no protective effect on serine esterase during heating (Fig. 6, ninth through fourteenth lanes). Control experiments confirmed that when unheated or heated alone, both Hsp16.6 and bovine serum albumin remain fully soluble, and only serine esterase showed heat-dependent insolubilization (data not shown; see also Fig. 7).

Protection of serine esterase by Hsp16.6 implies that, during heating, Hsp16.6 and serine esterase interact, forming a soluble Hsp16.6-serine esterase complex. To test for Hsp16.6-serine esterase interaction, 10 μM serine esterase and 20 μM Hsp16.6 either alone or mixed were incubated at room temperature or at 46 °C for 0, 20, or 60 min. The soluble fraction from the incubation mixtures was then separated by size-exclusion chromato-
order of one-twentieth of the amount of protein that becomes insoluble during the heat stress based on estimates that 5% of the total radioactivity becomes insoluble during heat stress. Thus, the actual difference in protein solubility between wild-type and Δhsp16.6 cells is expected to be small; and indeed, we have been unable to detect significant differences in the insoluble protein fraction from heat-stressed wild-type and Δhsp16.6 cells.3 The insoluble fraction of Hsp16.6 is most likely equally important for cell protection. Insolubilization of sHSPs during heat stress is observed in mammals, plants, and other prokaryotes (17, 28, 32–34), and there is evidence that this insolubilization is reversible. As indicated above, insolubilization could be explained by sHSP interaction with substrates at high sHSP/substrate ratios. Recent work indicates that insoluble sHSP-substrate aggregates are good in vitro substrates for the action of the chaperone ClpB in conjunction with the DnaK system (9). In vivo functional interaction of ClpB and sHSPs is observed as an increase in temperature sensitivity of sHSP and ClpB double mutants in both E. coli and Synechocystis (26, 28), as well as delayed resolubilization of sHSP-containing protein aggregates in ClpB mutants in E. coli (28) and in Hsp101 mutants in Arabidopsis.3 Furthermore, in E. coli, inclusion bodies containing sHSPs have been shown to be dynamic structures from which proteins can be resolubilized (35), and inclusion body formation and dissolution are affected by the presence of ClpB (36).

The diversity of cellular functions exhibited by Hsp16.6-associated proteins is similar to what has been observed for proteins reported to be substrates of DnaK (37) and GroEL (38). Although our sample size is small, putative Hsp16.6 substrates include a larger number of high molecular mass proteins than would be expected based on the mass distribution of all predicted open reading frames in the Synechocystis genome.4 We have not yet identified Hsp16.6-associated proteins over 60

TABLE I
Hsp16.6-associated proteins identified by MALDI-TOF mass spectrometry

| No. | Accession no. | Synecbocystis reading frame | Protein | Mass (Da) | pI | No. identified peptides | % Protein identified |
|-----|---------------|-----------------------------|---------|-----------|----|-----------------------|-------------------|
| 4   | P72749        | slr1105                     | TyPLA/BiPA GTPase | 66,013 | 4.98 | 8                     | 19                |
| 28  | P26327        | slr1329                     | ATP synthaseβ | 51,733 | 4.89 | 10                    | 35                |
| 3   | CAA63961      | slr1643                     | Ferredoxin-NADP+ reductase | 46,188 | 5.87 | 7                     | 23                |
| 5   | P73530        | slr1356                     | 30 S ribosomal protein S1(A) | 36,570 | 4.57 | 5                     | 23                |
| 9   | P73297        | sll1818                     | RNA polymerase α-subunit | 34,905 | 4.72 | 5                     | 23                |
| 2   | P72849        | sll1184                     | Heme oxygenase | 27,051 | 6.24 | 5                     | 19                |
| 1   | P74070        | sll1261                     | Elongation factor Ts | 24,231 | 5.37 | 6                     | 39                |
| 13  | S75304        | sll1294                     | Serine esterase | 22,210 | 5.08 | 3                     | 21                |
| 12  | P72955        | sll0643                     | UreG | 22,013 | 5.09 | 7                     | 48                |
| 27  | P72796        | sll1669                     | Shikimate kinase | 20,698 | 4.63 | 4                     | 30                |
| 6   | S75356        | slr2024                     | Response regulator | 20,233 | 6.34 | 5                     | 37                |
| 25  | S74880        | slr1251                     | Peptidyl-prolyl isomerase | 19,355 | 5.34 | 5                     | 28                |
| 15  | P74618        | slr0999                     | tRNA/rRNA methyltransferase | 17,033 | 5.64 | 4                     | 32                |

* Numbering as in Fig. 2B.

3 E. Basha and E. Vierling, unpublished data.
4 Available at www.kazusa.or.jp/cyano/cyano.html.
insoluble cell fraction. S1 is an unusual acidic ribosomal protein that cycles on and off the ribosome, binding mRNA and the 30 S subunit to promote translation initiation (42); and recent data indicate that S1 also binds RNase E and polynucleotide phosphorylase, possibly linking translation and mRNA degradation (43). *Synechocystis* S1 clearly functions in context with the ribosome based on the activity of more closely related homologs in chloroplasts (42) and other cyanobacteria (44).

Also involved in translation, elongation factor T9, a GDP/GTP exchange factor, is heat-labile and sHSP-associated. A third protein that could affect translational activity is a putative tRNA7tRNA methyltransferase. This protein has significant identity to *E. coli* SpoU (also called TrmH), which modifies RNA bases (45). The interaction of Hsp16.6 with S1 and a potential role in translation and/or RNA metabolism are similar to observations in eukaryotes, where sHSPs are associated with stress granules containing stalled translation initiation complexes (46).

Other sHSP-associated proteins represent a diversity of functions. Three proteins are enzymes of secondary metabolism: heme oxygenase (47), serine esterase, and shikimate kinase. Ferredoxin-NADP⁺ reductase is an essential protein that may function in multiple electron transport pathways (48) and, along with the membrane-associated β-subunit of ATP synthase, represents proteins essential for energy metabolism. Three proteins can be loosely grouped in signaling/protein modification, including a peptidyl-prolyl cis-trans isomerase, UreG (49), and a putative response regulator. A homolog of *E. coli* TypA/BipA, a poorly understood GTPase in the elongation factor G/Tu superfamily and a substrate of DnaK (37), is also associated with Hsp16.6. The function of TypA/BipA is unknown, but it is interesting that loss of function in *E. coli* leads to cold sensitivity (50). Heat sensitivity of this mutant has not been tested.

The Hsp16.6-associated proteins are heterogeneous with regard to their physical properties and structural characteristics. As is already clear, they span a range of molecular masses and pl values, and a survey of the atomic structures of proteins closely related to any of the substrates did not reveal any common features of secondary structure or domain packing that might be a signature for an sHSP substrate. Similarly, specific structural characteristics of DnaK and GroEL substrates are not clearly defined (37, 38). The current model for sHSP chaperone activity includes direct interaction of sHSP with substrate and improved reactivation of substrates denatured in the presence of the sHSP as opposed to the absence of the sHSP. Although all the proteins identified here co-immunoprecipitated with the sHSP and could be released by the ATP-dependent activity of the DnaK chaperone machinery, direct sHSP interaction with any of these proteins in *vivo* has not been proven. However, purified *Synechocystis* serine esterase proved to be heat-labile and to complex with Hsp16.6 in *vitro*. Furthermore, we note that purified serine esterase, shikimate kinase, and heme oxygenase are all difficult to work with due to tendency to aggregate, which is consistent with the importance of chaperones for maintaining their solubility under stress conditions. We have not been able to test for activity of these enzymes in whole cell extracts. Thus, it remains to be determined if the absence of Hsp16.6 affects the activity of these proteins after heat stress in the cell.

In addition to chaperone activity, sHSPs have recently been shown to be "membrane active" proteins in that purified sHSPs, including Hsp16.6, can modulate fluidity of isolated thylakoids and also model membranes (51, 52). How this prop-

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5 A. C. Hausrath and E. Vierling, unpublished data.
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property reflects sHSP activity in vivo is still unknown. It is notable that none of the substrates identified in our study correspond to photosynthetic membrane proteins, although these proteins represent major constituents of Synechocystis cells and would be solubilized under the conditions of our experiments.

It is now well established that all cells have a complex network of chaperones and proteases involved in protein quality control within the cell (55). The importance of sHSPs for protein quality control during heat stress appears to be highly variable in different organisms, as reflected in the minor phenotypes associated with hspAB deletions in E. coli and the lack of phenotype of sHSP deletions in Saccharomyces cerevisiae compared with the easily detected phenotype in Synechocystis. A reasonable explanation for this difference is that redundancy of functions within the quality control system varies between organisms and that a change in the balance of the different chaperone and protease systems may be required to reveal an sHSP-associated phenotype in many instances. Nonetheless, the sHSPs clearly can play a major role in stressed cells, and sHSP-associated phenotype in many instances. Nonetheless, the sHSPs clearly can play a major role in stressed cells, and

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