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Functional Mode of NtHSP17.6, a Cytosolic Small Heat-Shock Protein from *Nicotiana tabacum*

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Small heat-shock proteins (sHsps) are ubiquitous stress proteins with molecular chaperone activity. They share characteristic homology with the α-crystallin protein of the mammalian eye lens as well as being ATP-independent in their chaperone activity. We isolated a clone for a cytosolic class I sHsp, *NtHSP17.6*, from *Nicotiana tabacum*, and analyzed its functional mode for such activity. Following its transformation into *Escherichia coli* and its over-expression, NtHSP17.6 was purified and examined *in vitro*. This purified NtHSP17.6 exhibited typical chaperone activity in a light-scattering test. It was enable to protect a model substrate, firefly luciferase, from heat-induced aggregation. Non-denaturing PAGE showed that NtHSP17.6 formed a dodecamer in its native conformation, and was bound to its substrate under heat stress. A labeling test with bis-ANS indicated that this binding might be linked to newly exposed hydrophobic sites of the NtHSP17.6 complexes during heat shock. Based on these data, we suggest that NtHSP17.6 is a molecular chaperone that functions as a dodecamer in a heat-induced manner.

Keywords: in vitro activity, luciferase (Luc), molecular chaperone, small heat-shock protein (sHsp)

Plant stresses, in the form of heat, cold, dehydration, oxidation, or osmotic shock, can seriously damage cellular functioning and viability. Especially at high temperatures, many proteins tend to form aggregates devoid of biological activity (Jaenicke, 1995; Horwich and Weissman, 1997). To protect themselves from these threats, all organisms (including archea, bacteria, and eukarya) have developed mechanisms in which heat-shock proteins (Hsps) are synthesized (Narberhaus, 2002). Hsps are highly conserved protein families comprising five classes, according to their molecular weights: 110-kDa heat-shock protein (Hsp110), 90-kDa heat-shock protein (Hsp90), 70-kDa heat-shock protein (Hsp70), 60-kDa heat-shock protein (Hsp60), and a small heat-shock protein (sHsp) (Nover, 1990; Vierling, 1991; Cho and Hong, 2004).

All Hsps function as molecular chaperones (Jakob et al., 1993; Fink, 1999; Agashe and Hartl, 2000), which prevent the aggregation of unfolded polypeptides and assist in or lead to the correct re-folding of chaperone-bound denatured polypeptides in the cell under physiological and stress conditions (Jaenicke, 1995; Ellis, 1997; Fink, 1999; Haslbeck, 2000; Radford, 2000). The release of substrate is often coupled to an ATP-directed conformational change in the chaperone (Schlieker et al., 2002).

Plants are unique among eukaryotes in that they synthesize multiple sHsp gene families (Waters et al., 1996; Scharf et al., 2001). Whereas the Hsps in other organisms are found in the cytosol, plants express three types of cytosolic sHsps as well as specific isoforms targeted to intracellular organelles, such as the mitochondrion, chloroplast, and endoplasmic reticulum (Waters and Vierling, 1999). Members of the sHsp families share characteristic carboxy terminal sequences that share homology with the α-crystallin protein of the vertebrate eye lens (de Jong et al., 1993, 1998). Now that the three-dimensional crystal structures of sHsps have been revealed and their biochemical characteristics analyzed, it is clear that sHsps form large, multimeric complexes of 9 to 40 subunits, ranging in size from 200 to 800 kDa (Kim et al., 1998; van Montfort et al., 2002). These structures are believed to be necessary for functions within the cell (Waters et al., 1996; Smykal et al., 2000).

As molecular chaperones, the *in vitro* functioning of sHsps is of multimeric form. In early research, plant sHsps were shown to protect crude proteins from heat-induced insolubilization (Jinn et al., 1989). Current models propose that they function to prevent irreversible protein aggregation and insolubilization, thereby increasing the stress resistance of cells (Horwitz, 1992; Plesofsky-Vig and Brambl, 1995; van Montfort et al., 2002). It has been proposed that sHsps bind to their substrate proteins at denaturing temper-

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atures, and form a complex of folding intermediates in an ATP-independent fashion (Lee et al., 1997; van Montfort et al., 2002). Although the recognized range of substrates covers peptides as well as oligomeric enzymes (Ehrnsperger et al., 1997, 1998; Basha et al., 2004a), no common substrate characteristics have yet been suggested. Several studies support the fact that sHsps interact with their substrates through hydrophobic interactions, which can be increased due to newly exposed hydrophobic regions under heat-shock conditions (Das and Surewicz, 1995; Lee et al., 1997; Sharma et al., 1998). Even though sHsps exhibit this function through the binding of non-native proteins, it has been suggested that the substrates are released and reactivated with the help of other types of molecular chaperones in an ATP-dependent process (Lee et al., 1997; Veinger et al., 1998; Mogk et al., 1999, 2003; Haslbeck, 2000; Lee and Vierling, 2000).

We previously isolated seven genomic clones for the cytosolic class of sHsps from *Nicotiana tabacum*, and analyzed their individual expression patterns in response to abiotic stresses (Park, 2002). Early studies revealed that, among those clones, NtHSP18.2 and NtHSP18.3 protected a broad range of proteins from heat shock (Kim et al., 2004; Yu, 2004). In the present report, we show that another sHsp from tobacco, NtHSP17.6, also has *in vitro* chaperone activity.

MATERIALS AND METHODS

Genomic Clone *NtHSP17.6*

A tobacco genomic library in the EMBL3 arm has been screened with the ^{32}P-labeled *NtHSP18.2* cDNA clone (Joe et al., 2000), and seven genomic clones coding cytosolic class I sHsps have been isolated (Park, 2002). Here, we amplified an open reading frame (ORF) of one of those genomic clones, NtHSP17.6, using two primers that flanked this ORF: 5′ primer, 5′-ATATGAGCTCATGCTCCTGATTCCG AGC-3′, 3′ primer, 5-TATAGCATGCTTAAGCAGAGAT GTCAATG-3. *Sac*I and *Sph*I restriction sites, respectively underlined, were introduced into those primers. After heating to 95°C for 3 min, PCR was performed in a thermal cycler (Perkin-Elmer, USA) for 30 cycles (95°C, 30 s; 50°C, 30 s; and 72°C, 1 min). The amplified product was digested with *Sac*I and *Sph*I, and ligated into the pBAD expression vector (Kim et al., 2004) at the *Sac*I and *Sph*I cloning sites. The sequence of the cloned coding region was confirmed by dideoxy chain termination sequencing (Sanger et al., 1977), using Sequenase version 2.0 (United States Biochemical, USA).

Expression and Purification of NtHSP17.6 from *Escherichia coli*

The ORF of *NtHSP17.6* in the pBAD vector was transformed into *E. coli* strain MC1061, which was grown in 1.5 L of an LB medium at 37°C. Expression of NtHSP17.6 was induced by adding 0.125% of L(+)-arabinose (Lancaster, UK) for 16 h. Cells were harvested by centrifugation for 20 min at 4,500*g* (Kontron Instruments, Italy), and re-suspended in TE buffer (25 mM Tris-Cl, 1 mM EDTA, pH 7.5). The resuspended cell pellet was treated with 1 mM benzamidine, 5 mM ε-aminocaproic acid, and 2 mM PMSF; and then sonicated (total processing time, 10 min; pulse-on: 5 s; pulse-off: 55 s). The soluble fraction was separated by microcentrifugation for 20 min at 15,000 rpm, and precipitated with 60 to 95% ammonium sulfate (Sigma, USA). This precipitate was then re-suspended in 5 ml of TE buffer and dialyzed against 1 L of TE buffer. Afterward, the dialysate was applied to a 0.2 to 0.85 M sucrose density gradient (in TE linear) in a Beckman Quick-seal tube, followed by ultracentrifugation for 3 h at 50,000 rpm in a VC53 rotor (Beckman, USA). Fractions containing NtHSP17.6 were obtained and dialyzed against 2 L of TE buffer. The proteins were allowed to bind on a Q Sepharose fast flow column (Amersham Biosciences, USA) equilibrated in TE buffer, and then eluted with linear-concentration solutions of NaCl (from 40 to 400 mM) in TE buffer. Fractions containing NtHSP17.6 were again collected and dialyzed against 1 L of TE buffer. After incubation in 3 M urea (Sigma) for 15 min at room temperature, the fractions were applied to a Q Sepharose fast flow column, and again eluted with TE buffer containing 40 to 400 mM of NaCl. Fractions with NtHSP17.6 at > 95% purity were pooled and dialyzed three times (12 h each) against 1 L of TE buffer containing 1 mM dithiothreitol, then concentrated to 1 to 2 $\mu g\ \mu L^{-1}$ with Amicon centrifugal filters (Millipore, USA). Purified NtHSP17.6 was stored at −70°C in the presence of 50 mM KCl and 20% glycerol.

Light-Scattering Test

A measurement of light-scattering was used to quantify changes in the level of protein aggregation. Two hundred nM luciferase (Luc; Sigma) was heated to 42°C with NtHSP17.6 (200 nM to 1 μM) in a total

volume of 500 µl that contained 25 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 25 mM KCl. Absorbance was monitored every 2.5 min at 340 nm in a spectrophotometer (Spectronic Instruments, USA).

Formation of Expanded Complex between NtHSP 17.6 and Luciferase

Five µM of Luc was incubated for 40 min with purified NtHSP17.6 (5 to 15 µM) at 25 or 42°C, in a total volume of 30 µl. The samples were centrifuged for 20 min at 16,250*g*; and the supernatant fractions were applied to non-denaturing PAGE, and the pellet fractions were applied to SDS-PAGE.

Incorporation of bis-ANS to NtHSP17.6

1,1′-Bis(4-anilino)naphthalene-5,5′-disulfonic acid (bis-ANS) was allowed to incorporate into NtHSP17.6 so that we could investigate the exposure of hydrophobic domains in NtHSP17.6 after heating. Twenty µg of NtHSP17.6 was incubated at either 25°C or 42°C for 1 h in 500 µl of 25 mM Tris-Cl (pH 7.5), 100 mM KCl, and 10 µM of bis-ANS. Afterward, the bis-ANS was excited at 390 nm and its fluorescence was scanned from 400 to 600 nm in a fluorescence spectrometer (JASCO, UK).

Polyacrylamide Gel Electrophoresis

SDS-PAGE was conducted on 15% (w/v) acrylamide gels (Anderson et al., 1972; Sambrook et al., 1989), while non-denaturing PAGE was performed on 4 to 22.5% (w/v) acrylamide gradient gels, as described by Anderson et al. (1972). The size markers for the former were from the BenchMark Protein Ladder (Invitrogen, USA), while those for the non-denaturing PAGE were either bovine serum albumin (BSA; Sigma) for 66 kDa (monomer) and 132 kDa (dimer), or jack bean urease (Sigma) for 272 kDa (trimer) and 545 kDa (hexamer).

RESULTS

NtNSP17.6 Expressed and Purified from *E. coli*

NtHSP17.6 was over-expressed in *E. coli* by induction with arabinose, and was purified through a process of ammonium sulfate precipitation, sucrose density gradient, and anion exchange chromatography, with and without detergent treatments. The over-expressing NtHSP17.6 remained soluble, and accounted for approximately 2% of the total *E. coli* proteins (Fig. 1). Most of NtHSP17.6 was precipitated in 60 to 95% ammonium sulfate. When applied to the 0.2 to 0.85 M sucrose density gradient, NtHSP17.6 was separated to a fraction of high-sucrose concentrations close to 0.85 M. From our first anion exchange chromatography without detergent, NtHSP17.6 was mainly eluted in 220 to 260 mM NaCl solutions. From the second anion exchange chromatography with the 3 M urea treatment, NtHSP17.6 was mainly eluted in 180 to 200 mM NaCl solutions. When purified NtHSP17.6 was dialyzed thoroughly in TE buffer with 1 mM DTT, it was recovered at > 98% purity. The apparent molecular mass of purified NtHSP17.6 was about 17.6 kDa.

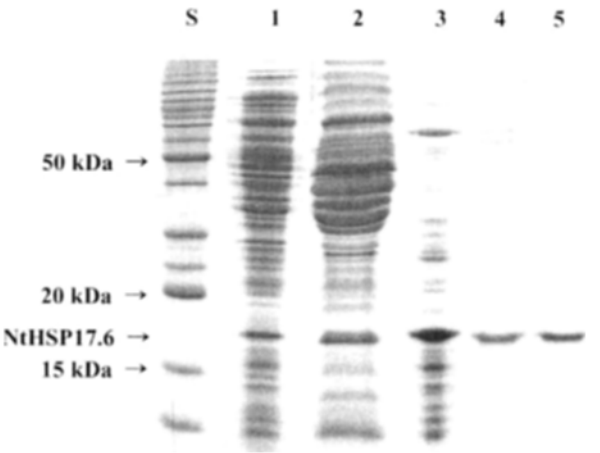

Figure 1. SDS-PAGE of NtHSP17.6 during purification process. Samples from each step were analyzed on 15% SDS-PAGE gels. Lane 1, after induction with L(+)-arabinose, total *E. coli* cellular proteins contained over-expressed NtHSP17.6. Lane 2, NtHSP17.6 was precipitated in 60 to 95% ammonium sulfate. Lane 3, NtHSP17.6-enriched fractions pooled from 0.2 to 0.85 M sucrose density gradient centrifugation. Lane 4, eluted NtHSP17.6 from first anion exchange chromatography. Lane 5, eluted NtHSP17.6 after second anion exchange chromatography. S, standard size marker.

NtHSP17.6 Protects Luciferase from Aggregation

Firefly luciferase (Luc), a 61-kDa monomeric protein that is inactivated at moderate temperatures (39 to 42°C), is used for chaperone studies (Schumacher et al., 1994; Levy et al., 1995; Lee and Vierling, 2000). To investigate the *in vitro* molecular chaperone activity of NtHSP17.6, we used Luc to perform a light-scattering test. When 0.2 µM of Luc was exposed to 42°C, it aggregated rapidly, as evidenced by light-scat-

tering measured at 340 nm. Aggregation was saturated in approximately 25 min after heating. As NtHSP17.6 was added, the thermal aggregation of Luc decreased (Fig. 2). This chaperone activity was especially prominent at a 1:2 molar ratio of NtHSP17.6 to Luc. Above that ratio, NtHSP17.6 activity started to show a saturation effect. In contrast, when equivalent weights of BSA to Luc were used, more protein was aggregated, demonstrating the specific activity of NtHSP17.6 as a molecular chaperone. However, NtHSP17.6 itself did not manifest any noticeable aggregation under these heat-shock conditions (Fig. 2).

Heat-Induced Formation of Expanded Complexes between NtHSP17.6 and Luciferase

To evaluate the complexes of NtHSP17.6 and Luc under stress, luciferase was incubated at various molar ratios of Luc to NtHSP17.6, with or without heat shock. After the samples were centrifuged, the supernatant fractions were analyzed on a non-denaturing PAGE gel, while the pellet fractions were run on an SDS-PAGE gel (Fig. 3). We confirmed via non-denaturing native PAGE that NtHSP17.6 forms a dodecameric complex under both untreated and heat-shocked conditions. For unheated samples at 25°C, almost all the proteins were located in the supernatants, and Luc showed no significant aggregation. At the same time, NtHSP17.6 did not interact with Luc, even at high ratios. For heated samples at 42°C, Luc was not found in the supernatant but in the pellet in the absence of NtHSP17.6. However, in the presence

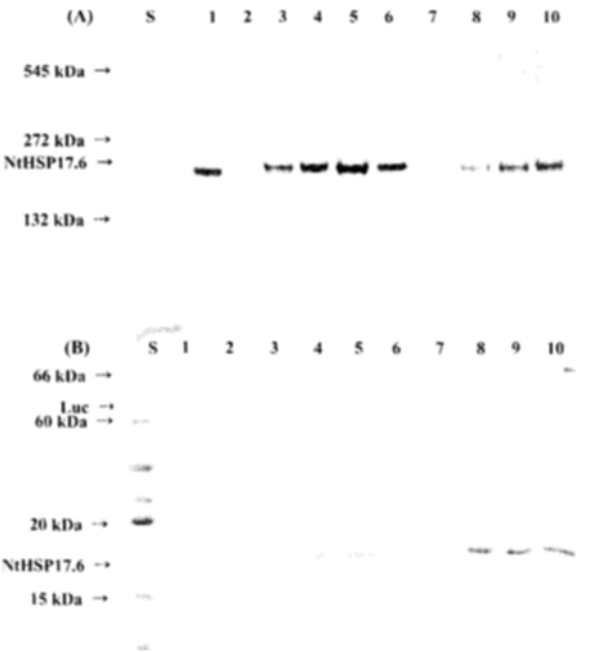

Figure 3. NtHSP17.6 formed expanded complexes with Luc and protected it from aggregation under heat shock. Luc (5 μM) was incubated with various concentrations of NtHSP17.6 (0.0 to 1.5 μM), at either 25°C or 42°C. After incubation for 40 min and centrifugation, supernatants and pellets were analyzed on 4 to 22.5% native PAGE gels **(A)** or 15% SDS-PAGE gel **(B)**, respectively. S, standard size marker; Lane 1, 10 μM of NtHSP17.6 incubated at 25°C; Lanes 2 to 5, 5 μM of Luc incubated with 0, 5, 10, or 15 μM of NtHSP17.6 at 25°C; Lane 6, 10 μM of NtHSP17.6 incubated at 42°C; Lanes 7 to 10, 5 μM of Luc incubated with 0, 5, 10, or 15 μM NtHSP17.6 at 42°C.

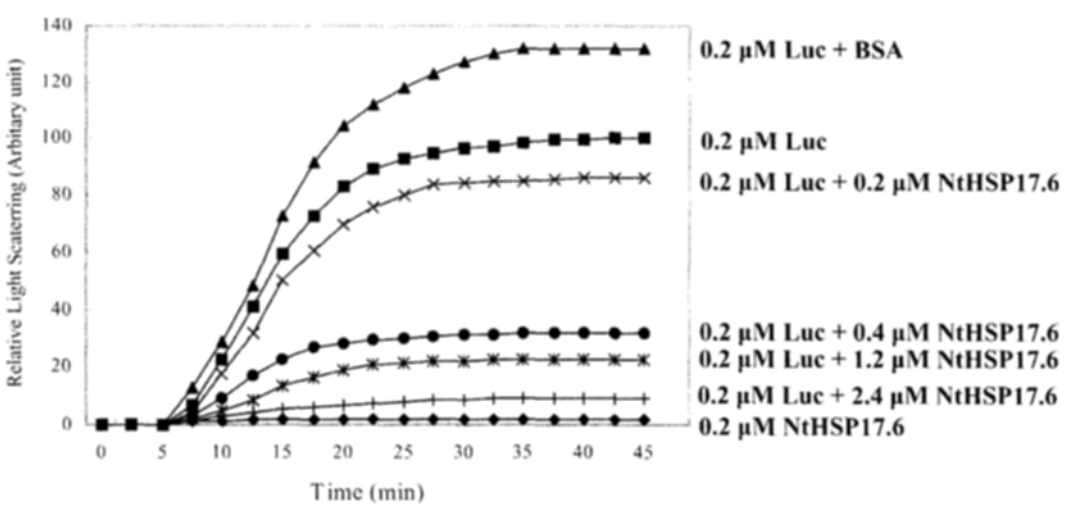

Figure 2. In light-scattering test, NtHSP17.6 prevented aggregation of luciferase. Luc (0.2 μM) was incubated at 42°C in absence of NtHSP17.6 or in presence of increasing concentrations of NtHSP17.6 (0.2, 0.4, 1.2, or 2.4 μM). Equivalent weight of BSA to Luc was used instead of NtHSP17.6 as negative control.

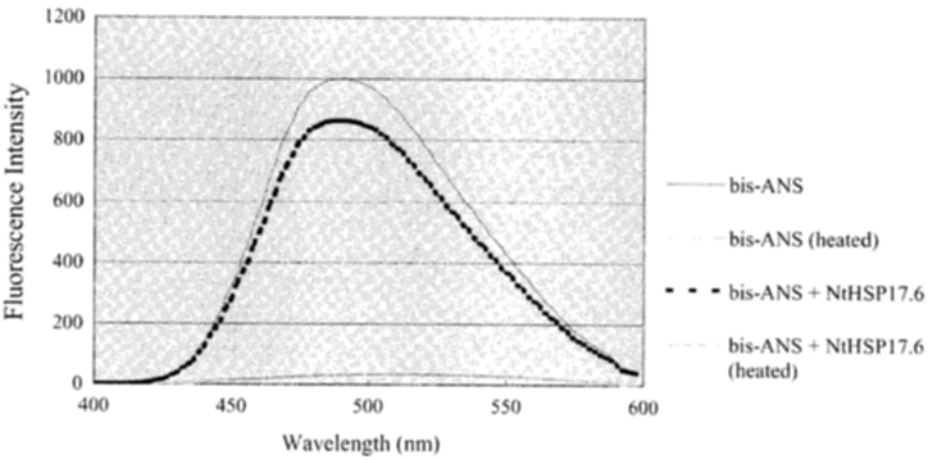

Figure 4. Incorporated bis-ANS was used to detect change in hydrophobicity of NtHSP17.6 during heating. NtHSP17.6 (20 μg) was incubated with 10 μM of bis-ANS at 25°C or 42°C for 1 h. Emitted fluorescence was measured after excitation of bis-ANS in fluorescence spectrophotometer.

of higher concentrations of this sHsp, less Luc was aggregated and more appeared in the supernatant than had been detected on the native PAGE gel. It was also evident that NtHSP17.6 interacted with Luc to form expanded complexes; i.e., a smearing signal was detected above the band for the NtHSP17.6 dodecamer complex (mainly from Lanes 8 to 10 in Fig. 3A), and more complexes were formed as higher amounts of NtHSP17.6 were added to the Luc solutions.

Incorporation of bis-ANS to Newly Exposed Hydrophobic Regions of NtHSP17.6 during Heat Shock

The fluorescent probe, 1,1′-bis(4-anilino)naphthalene-5,5′-disulfonic acid (bis-ANS), has been used extensively to demonstrate the presence of hydrophobic sites on the surfaces of proteins (Das and Surewicz, 1995; Sharma et al., 1998). To observe changes in the hydrophobicity of NtHSP17.6 before and after heat shock, we used fluorescence spectrophotometry to examine the amount of bis-ANS incorporated into that sHsp. In the absence of NtHSP17.6, bis-ANS itself showed a minimal level of fluorescence, with no apparent difference due to the heat-shock treatment. However, when incubated with NtHSP17.6 at 25°C, the degree of fluorescence increased dramatically, peaking at about 492 nm. After heating to 42°C, the fluorescence intensity rose further, by about 16%, compared with the unheated sample, and some blue shift was observed in the emission peak. This demonstrates that the hydrophobicity of NtHSP17.6 is increased under heat, and might result from the exposure of new hydrophobic sites in NtHSP17.6, which were hidden before heating (Fig. 4).

DISCUSSION

Small heat-shock proteins belong to a ubiquitous protein family with molecular chaperone activity. Its members share a conserved α-crystallin domain in the carboxy terminal, and show diversity in the amino terminal (Caspers et al., 1995). Studies of sHsps from various organisms have shown that they bind to their substrates stably and protect them, in an ATP-independent manner, from thermal or chemical aggregation (Ehrnsperger et al., 1997; Wang and Spector, 2000; Abdulle et al., 2002; Stromer et al. 2003; Basha et al., 2004b; Fu and Chang, 2004; Kim et al., 2004). This chaperone activity is likely related to the stress tolerance of cells (Joe et al., 2000; Park and Hong, 2002).

Since we first isolated seven genomic clones for cytosolic Class I sHsps from tobacco, we have attempted to compare their chaperone activities. Previously, we analyzed the functional modes of NtHSP18.2 and NtHSP18.3 (Kim et al., 2004; Yu, 2004). Here, we describe the *in vitro* chaperone activity of NtHSP17.6, another tobacco sHsp.

NtHSP17.6 was over-expressed in *E. coli* and purified (Fig. 1). The formation of multimeric complexes of small heat-shock proteins has been reported previously (Kim et al., 1998; van Montfort et al., 2002), and we also showed here that, under both heat-shocked and untreated conditions, NtHSP17.6 was likely to form a dodecamer with a molecular weight of about 210 kDa (Fig. 3). Even in the presence of our

Luc substrate, the size of the NtHSP17.6 complex did not change without any applied heat. This demonstrates that, unless the substrate was denatured, the sHsp did not bind to its substrate. However, under high temperatures, larger complexes developed, probably from a formation between the NtHSP17.6 complex and the heat-denatured luciferase (Fig. 3A).

This binding was directly linked to the ability of the sHsp to protect the substrate against irreversible aggregation. Our light-scattering assay showed that luciferase was rapidly aggregated at 42°C in the absence of the sHsp. However, as the concentration of NtHSP17.6 rose, relative light-scattering was more effectively decreased (Fig. 2). We compared this molecular chaperone activity of NtHSP17.6 to the enhancement effect of BSA additions in light-scattering, a phenomenon probably common to most proteins that lack the chaperone function. NtHSP17.6 was able to reduce relative light-scattering to about 10%, a result consistent with those from our PAGE gels. The decreased relative light-scattering values were coupled to the greater solubility of Luc (Fig 3). Similarly, this interaction between NtHSP17.6 and Luc required no supplemental ATP, as is also true with other sHsps (van Montfort et al., 2002). Although we added no ATP to any of our samples for either the light-scattering assay or the experiments to monitor expanded complex formation, still NtHSP17.6 exhibited chaperone activity to protect Luc.

The current model suggests that sHsps recognize hydrophobic sites of non-native proteins that are hidden under non-heat-shocked conditions (Ehrnsperger et al., 1999; Haslbeck et al., 1999; Haslbeck, 2000; van Montfort et al., 2002). High-temperature treatments induce changes in the conformation of sHsps to exposed hydrophobic residues, which interact with the hydrophobic regions of substrates. Because NtHSP17.6 generally is a hydrophilic as well as a soluble cytosolic protein, we used bis-ANS to investigate the change in sHsp hydrophobicity after heat shock (Fig. 4). The extent of bis-ANS binding to NtHSP17.6 detected by fluorescence implied some interaction existed between bis-ANS and NtHSP17.6 at 25°C. However, when the temperature was raised to 42°C, hydrophobicity of the NtHSP17.6 complexes increased (Fig. 4). It is likely that certain hydrophobic residues, which remain unknown, were newly exposed because of heat-induced conformational changes in those complexes.

Previous research on RNA blot hybridization of NtHSP17.6 from tobacco indicated that this sHsp is not expressed as highly as NtHSP18.2 or NtHSP18.3 during mild heat stress, but its expression is greatly increased under severe stress, e.g., at 48°C (Park, 2002). When compared with NtHSP18.3 (Yu, 2004), the strong molecular chaperone activity by NtHSP17.6 is more effective in preventing the aggregation of Luc at lower molar ratios. Furthermore, its increased expression level under severe heat stress suggests an important *in vivo* role as a molecular chaperone under extremely high temperatures.

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