A 21-kDa Chloroplast Heat Shock Protein Assembles into High Molecular Weight Complexes in Vivo and in Organelle*

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The conservation of the carboxyl-terminal “heat shock” domain among small (sm) cytoplasmic and chloroplast heat shock proteins (HSPs) suggests that these smHSPs perform similar functions. Previous studies have established that cytoplasmic smHSPs are found in higher order structures in vivo (approximately 500 kDa). To determine if the chloroplast smHSP is found in similar complexes, we examined the size of the 21-kDa chloroplast smHSP from Pisum sativum, PsHSP21, under non-denaturing conditions. Following sedimentation of chloroplast stromal extracts on sucrose gradients PsHSP21 is detected in fractions corresponding to 10–11 S. Upon non-denaturing gel electrophoresis, PsHSP21 was detected in two high molecular mass complexes of approximately 230 and 200 kDa, in good agreement with the sucrose gradient data. These PsHSP21-containing particles were stable under different salt and Mg2+ conditions, and their integrity was not affected by 1.0% Triton X-100 or 10 mM ATP. To study assembly of the high molecular weight complexes containing PsHSP21, in vitro translated PsHSP21 was imported into chloroplasts and its size was examined. Following import into chloroplasts isolated from heat-stressed plants, greater than 50% of PsHSP21 was recovered in the higher molecular weight forms. In contrast, following import into chloroplasts isolated from control plants the protein was recovered exclusively in a 5 S (approximately 42-kDa) form. These data suggest that preexisting PsHSP21 or other heat-induced factors may be required for assembly of the higher molecular weight particles. We propose that the 10–11 S particles are the functional form of PsHSP21.

Plants, like all other organisms, respond to high temperature by synthesizing a new group of proteins designated as heat shock proteins (HSPs)1 (1). The most abundant HSPs in plants are a group of small HSPs (smHSPs) which range in size from 15 to 30 kDa. Plant smHSPs belong to four multigene families and are localized to different cellular compartments including the chloroplast, cytoplasm, and endoplasmic reticulum (2–4). Plant smHSPs exhibit a conserved boxyl-terminal “heat shock domain” (approximately 80 amino acids) that is also found in all other eukaryotic smHSPs, as well as in the vertebrate α-crystallin eye lens proteins (1, 5).

Structural studies of cytoplasmic smHSPs and α-crystallins indicate that these proteins exist in higher order structures in vivo. Arrigo and Welch (6) estimated that human HSP28 has a native molecular mass of 500 kDa, and recombinant murine HSP25 has been modeled as a 730-kDa complex composed of 32 HSP25 monomers (7). The native sizes of Saccharomyces cerevisiae and Drosophila smHSPs have similarly been estimated to be on the order of 500 kDa, as determined by sucrose gradient or gel filtration analysis (8–10). Plant cytoplasmic smHSPs have been reported in complexes of 500 kDa (11) and more recently in significantly smaller sized particles of approximately 240 kDa (12). Chicken HSP24 is also found in a smaller complex (180 kDa) (13). The α-crystallins are isolated in particles in the same size range (800 kDa) or a smaller form (300 kDa), which is suggested to represent the inner core of the 800-kDa complex (14). Interestingly, HSP27 and α-crystallin have also been detected in the same 700-kDa complex in rat kidney cells (15). It has been hypothesized that the conserved heat shock domain is responsible for the similar structural properties of these proteins (5).

The correlation between expression of smHSPs and increased cellular thermotolerance has led to the hypothesis that smHSPs protect cells from heat-induced damage (1). When mammalian or Drosophila smHSPs have been overexpressed in mammalian cell lines, some increase in thermotolerance has been observed, providing additional evidence for a specific role of smHSPs in the thermotolerance phenomenon (16–18). However, deletion or overexpression of S. cerevisiae HSP26, the only identified smHSP in this organism, failed to provide evidence for an essential role of smHSPs during stress (19, 20). Thus the function of these proteins during heat stress remains poorly defined.

Although a number of functions have been proposed for the smHSPs (1, 11), recent experiments with both the α-crystallin proteins and mammalian smHSPs have suggested that these proteins function as “molecular chaperones” (21). Molecular chaperones are proteins that alter or maintain the structure of other polypeptides and thereby facilitate proper protein folding (22–25). A recombinant mammalian smHSP prevented heat-induced aggregation of other proteins in vitro, as assessed by light scattering, and increased the half-time of heat-induced inactivation of the enzyme α-glucosidase (22). It also increased the yield of active enzyme after dilution from denaturant (22). The mechanism of these effects remains unknown.

An smHSP (HSP21) that localizes to chloroplasts has been identified in many plant species (4, 26–31). It is a nuclear-encoded protein that is posttranslationally transported into chloroplasts. Comparative sequence analysis of the chloroplast smHSP from several species has defined both the conserved heat shock domain and a “methionine bristle” domain (28 amino acids) that is unique to the chloroplast smHSP (31).

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1The abbreviations used are: HSP, heat shock protein; smHSP, small heat shock protein; PAGE, polyacrylamide gel electrophoresis; Rubisco, ribulose-bisphosphate carboxylase.

13216

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Chloroplast smHSP mRNA is not found in unstressed plants but accumulates dramatically during heat shock (4, 32). The HSP21 protein increases correspondingly during stress to an estimated 0.02% of total leaf protein. Following stress, the smHSP is very stable, having a half-life of 52 h (32). Under moderate stress conditions, the majority of the protein is recovered from the soluble protein fraction of the chloroplast (32).

The conservation of the heat shock domain among cytoplasmic and chloroplast smHSPs suggests that these proteins serve similar functions in different cellular compartments. We sought to determine if the chloroplast smHSP from Pisum sativum (pea), PsHSP21, is also a component of a larger protein complex, similar to the previously described cytoplasmic smHSP complexes. By examining PsHSP21 in chloroplast extracts, as well as protein imported into isolated intact chloroplasts, we demonstrate that this smHSP is found in complexes of approximately 200–230 kDa. These results further support the hypothesis that there is functional homology between the cytoplasmic and chloroplast smHSPs.

MATERIALS AND METHODS

Plant Growth and Heat Shock Treatment—Pea seeds (P. sativum, cv. "Little marvel") were germinated in flats of vermiculite and grown in a growth chamber under either of the two conditions: (1) in a growth chamber with 22 °C day/18 °C night temperatures at a light intensity of 240 µmol m−2 s−1 (photosynthetically active radiation), and a 16-h photoperiod; or (2) on lighted shelves at 23–25 °C, at a light intensity of 120 µmol m−2 s−1, and a 16-h photoperiod. No differences were noted in experiments using plants grown under the two conditions. All plants were watered with 1/4 strength Hoagland's solution and grown for 9 days. Unless otherwise noted, heat stress was performed on intact plants at 38 °C according to the heat stress regime described previously (32). Briefly, the growth chamber temperature was increased gradually (4 °CAI) to 38 °C, maintained at 38 °C for 4 h, and decreased to the control temperature (22 °C) at the same rate.

Chloroplast Isolation and Fractionation—Intact chloroplasts were isolated from control or heat-stressed plants using Percoll gradients as previously described (27, 32). When using heat-stressed plants, chloroplasts were isolated either at the end of the 4-h maximal temperature (38 °C) stress for in vitro native structure studies, or 18 h after the end of the heat shock regime for import experiments unless otherwise indicated. Chloroplasts were lysed in buffer containing 10 mM HEPES, pH 8.0, 1.0 mM 2-mercaptoethanol, 5 mM MgCl2, 150 mM NaCl, and protease inhibitors (5.0 mM e-aminocaproic acid, 1.0 mM benzamidine, 1.0 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml leupeptin A, and 0.5 mg ml−1 each antipain, aprotinin, chymostatin, and leupeptin). Variations in the lysis buffer are detailed in the results and corresponding figure legends. The chloroplast lysate was spun for 10 min at 12,000 × g for 15 min, and the supernatants corresponding to approximately 1.5 mg (fresh weight) to 6.5 g chlorophyll and then frozen directly on the gel. When necessary, Triton X-100 was removed from the protein fraction prior to electrophoresis by passage through Sephadex G-25 (0.2-ml column bed volume) equilibrated in the standard lysis buffer (34). Following SDS-PAGE or native gel electrophoresis, either the gels were stained with Coomassie Brilliant Blue R, fixed, and exposed to x-ray film or proteins were transferred to nitrocellulose membrane filters for immune detection of PsHSP21. [35S]Met-labeled PaHSP21 from in vitro import reactions was quantified by determining the radioactivity of PsHSP21 bands on the dried gels with a Betagen analyzer (Betalen). The counts/min per cpm per band was typically 65–80, with a background of 1.0–1.5 cpm. Immunoblots were reacted with antisera against PsHSP21, which is specific for the carboxyl-terminal segment of the protein (amino acids 131–232) as characterized previously (36). Antibody reactions were performed as described (32) using a 1:5000 dilution of PaHSP21 antisera, and antibodies were detected with 125I-protein A (46.6 µCi µg−1; ICN Radiochemicals) or using chemiluminescence (ECL; Amersham Corp.). Western blotting results were quantified by counting the 125I-labeled bands excised from the nitrocellulose filters (32). The range of cpm per excised band was 800–1000 cpm. In some experiments, proteins from chloroplast membrane fractions were also analyzed by SDS-PAGE and immunoblotting as for proteins from the soluble fractions.

RESULTS

PsHSP21 Is Found in High Molecular Weight Complexes in Vivo—Our previous studies have shown that, when heat stress is applied gradually and light intensity is moderate (250–350 µmol m−2 s−1), the majority of PsHSP21 is localized in the soluble protein fraction of the chloroplast (32). In order to examine the native size of soluble PsHSP21, total soluble chloroplast proteins from control or heat-stressed pea plants were separated on 17–34% sucrose gradients. Because the HSP21 polypeptide was removed from the lysed chloroplasts by centrifugation for 10–15 min at 12,000 × g and the soluble proteins were further analyzed as described below.

Sucrose Gradient Centrifugation—Sucrose gradients (17–34% sucrose) containing the same components as the chloroplast lysis buffer were used to estimate the sedimentation coefficient of PsHSP21. For in vitro studies, the soluble protein fraction corresponding to 500–800 µg of chlorophyll was loaded onto the gradient and centrifuged at 4 °C for 21 h at 40,000 × g in a Beckman SW 40 rotor. For analysis of in vitro import reactions, soluble protein corresponding to 80–100 µg of chlorophyll was fractionated on each gradient. After centrifugation, 12-ml fractions were collected and proteins from each fraction, were analyzed either by SDS-PAGE and immunoblotting for in vitro studies, or by SDS-PAGE and fluorography for in vitro import studies. For in vitro studies, 60 µl of each gradient fraction was loaded on the gel. For in vitro studies, proteins from each fraction were precipitated with trichloroacetic acid and washed with 80% acetone, after which 25% of each gradient fraction was loaded on the gel.

Acrylamide Gel Electrophoresis and Immunoblotting—For analysis by SDS-PAGE, protein samples from the soluble fraction of chloroplasts or from sucrose gradients were mixed directly with 2 x SDS gel sample buffer (1 x buffer is 60 mM Tris-HCl, pH 8.0, 60 mM dithiothreitol, 2% SDS, 5.0 mM e-aminocaproic acid, 1.0 mM benzamide, 15% sucrose) or were concentrated as above from trichloroacetic acid precipitation before resuspension in 1 x SDS gel sample buffer. Samples were boiled for 2–3 min and separated by electrophoresis on 12.5% SDS-polyacrylamide gels (34). Following SDS-PAGE or native gel electrophoresis, either the gels were stained with Coomassie Brilliant Blue R, fixed, and exposed to x-ray film or proteins were transferred to nitrocellulose membrane filters for immune detection of PsHSP21. [35S]Met-labeled PaHSP21 from in vitro import reactions was quantified by determining the radioactivity of PsHSP21 bands on the dried gels with a Betagen analyzer (Betalen). The counts/min per cpm per band was typically 65–80, with a background of 1.0–1.5 cpm. Immunoblots were reacted with antisera against PsHSP21, which is specific for the carboxyl-terminal segment of the protein (amino acids 131–232) as characterized previously (36). Antibody reactions were performed as described (32) using a 1:5000 dilution of PsHSP21 antisera, and antibodies were detected with 125I-protein A (46.6 µCi µg−1; ICN Radiochemicals) or using chemiluminescence (ECL; Amersham Corp.). Western blotting results were quantified by counting the 125I-labeled bands excised from the nitrocellulose filters (32). The range of cpm per excised band was 800–1000 cpm. In some experiments, proteins from chloroplast membrane fractions were also analyzed by SDS-PAGE and immunoblotting as for proteins from the soluble fractions.

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Chloroplast HSP Native Structure

Fig. 1. Chloroplast PsHSP21 from heat-stressed plants sediments at 10-11 S. The soluble proteins from isolated chloroplasts were sedimented on 17-34% sucrose gradients and fractions were analyzed by SDS-PAGE. Gels were stained with Coomassie Blue (panel A) or blotted to nitrocellulose and reacted with PsHSP21 antibodies (panel B). Antibody detection was with 125I-protein A. Lanes 1-12, sucrose gradient fractions from top to bottom of the gradient. P, pellet from sucrose gradient. Molecular size standards for the SDS-PAGE are labeled at right (in kDa). Position of protein markers used as sedimentation standards are shown above the corresponding fractions. Asterisk in panel B indicates the position of the 42-kDa polypeptide discussed under “Results.”

Fig. 2. PsHSP21 is detected in two particles of approximately 200 and 230 kDa on pore size exclusion non-denaturing gels. Lane 1, chloroplast-soluble proteins from control plants; lane 2, chloroplast soluble proteins from heat-stressed plants; lane 3, total leaf proteins from heat-stressed plants; lane 4, total root proteins from heat-stressed plants. Following electrophoresis gels were blotted to nitrocellulose and reacted with PsHSP21 antibodies. Antibodies were detected using chemiluminescence. Position and sizes of molecular mass standards are indicated at the right. Arrows at the left mark the location of the 200- and 230-kDa forms of PsHSP21.

is not sufficiently abundant to be detected by Coomassie Blue or silver staining of chloroplast extracts, gradient fractions were analyzed by SDS-PAGE and Western blotting using PsHSP21 antibodies (Fig. 1). When soluble proteins from heat-stressed and control (not shown) plants were compared, no major differences were detected in the stained gels. Western blot analysis of the gradient fractions showed that the majority (77.3%) of PsHSP21 sedimented in fractions 4-7 (21-27.5% sucrose) with the peak (57%) in fractions 5 and 6 (24.5-26% sucrose) (Fig. 1B). Less than 2% of PsHSP21 was recovered in the pellet fraction of the gradient. Using bovine serum albumin (6 S), catalase (11 S), and ribulose-bisphosphate carboxylase (Rubisco) (18 S) as standards on the gradients, a sedimentation value of 10-11 S was estimated for the fractions containing PsHSP21. For a globular protein, this value corresponds to an average molecular mass of approximately 200 kDa. Other than PsHSP21, an additional protein band of 42 kDa was also recognized by the PsHSP21 antibodies (Fig. 1B). The abundance of this band in each fraction was always proportional to that of PsHSP21 (Fig. 1B), suggesting it is a dimeric form of PsHSP21 that has not been denatured by the SDS sample buffer.

Non-denaturing gel electrophoresis was used as an independent approach to examine the size of PsHSP21 in chloroplast extracts. Soluble proteins from chloroplasts isolated from heat-stressed or control plants were prepared and analyzed on pore exclusion non-denaturing gradient gels (35). PsHSP21 was identified by Western blotting with PsHSP21 antiserum (Fig. 2), and the size of the reacting bands was determined relative to molecular mass markers. On these gels the mass of globular proteins can be estimated relative to molecular mass standards because proteins migrate to their exclusion limit (35). In the heat shock chloroplast sample, PsHSP21 antiserum reacted strongly with two protein bands with apparent masses of 200 and 230 kDa. These bands were not detected in the control samples, consistent with the absence of HSP21 in control plants (32). The apparent mass of these major bands is consistent with the sedimentation data but provides higher resolution and suggests that the 10-11 S form consists of at least two molecular mass species. To verify that the major 200- and 230-kDa bands contained HSP21, a two-dimensional separation was performed. A heat shock chloroplast sample was first separated as shown in Fig. 2. The sample lane was excised from the gel and equilibrated in denaturing SDS sample buffer and placed lengthwise on top of an SDS gel for a second dimension separation. The second dimension gel was then blotted to nitrocellulose and reacted with PsHSP21 antiserum. The antiserum detected a 21-kDa polypeptide at positions corresponding to the major 200- and 230-kDa species, indicating both high molecular weight complexes contain PsHSP21 (not shown).

We have previously shown that chloroplast PsHSP21 is also expressed in roots during heat stress (32). To determine if PsHSP21 is found in a similar large complex in non-photosynthetic tissues, proteins isolated from total root or total leaf tissues were also analyzed by non-denaturing gel electrophoresis. The same 200- and 230-kDa bands were detected in protein extracts prepared from either total root or leaf tissues of heat...
shocked plants (Fig. 2). Therefore, the formation of the high molecular weight complexes containing PsHSP21 is not specific to photosynthetic organelles, but is a process occurring in all types of plastids. These results also indicate that particle formation is not an artifact of the chloroplast isolation procedure, since the particles are found in whole tissue extracts as well. The total leaf and root extracts also behaved identically to chloroplast proteins by sucrose gradient analysis (Table I). None of the conditions caused apparent dissociation of the 10-11 S form or increased NaCl and MgCl₂, or addition of EDTA, reducing agent, detergent (1% Triton X-100), and ATP. For the sucrose gradient analyses, the buffer components in the lysis and gradient solutions were identical. As seen in Table I, under all conditions tested, approximately 50% of PsHSP21 was detected in fractions sedimenting between 10 and 11 S (24.5-26% sucrose). None of the conditions caused apparent dissociation of the 10-11 S form or increased the fraction of PsHSP21 in the gradient pellet (<2%). We also saw no change in protein distribution if chloroplasts were isolated the morning following the day of stress (24-h sample, Table I).

Analysis by non-denaturing gel electrophoresis of the soluble proteins from chloroplasts lysed under the same conditions listed in Table I is shown in Fig. 3. Chloroplasts were incubated in all lysis solutions for 1 h at 4°C before removal of membranes, with the exception of the samples containing Triton X-100, in which the detergent was added to the soluble fraction after membranes were pelleted. None of the conditions tested altered the pattern of immunoreactive bands; both the 230- and 200-kDa forms were detected in all samples at approximately the same ratio. The similar sedimentation and electrophoretic behavior of PsHSP21 under all of these conditions supports the conclusion that the high molecular weight forms are stable native complexes containing PsHSP21.

### Table 1

| Sample | Lysis and gradient buffer | Distribution in gradient (%) |
|--------|----------------------------|----------------------------|
| Chloroplasts | Standard buffer | 79 | 52 |
| 0 mm NaCl | 82 | 65 |
| 500 mm NaCl | 73 | 49 |
| 500 mm NaCl, 20 mm MgCl₂ | 76 | 51 |
| 0 mm MgCl₂, 0.75 mm EDTA | 72 | 48 |
| 0.75 mm EDTA | 72 | 48 |
| 0 mm NaCl, 150 mm KCl | 68 | 60 |
| + 20 mm DTT | 75 | 49 |
| + 10 mm ATP | 70<sup>d</sup> | 50<sup>d</sup> |
| + 1% Triton X-100 | 70<sup>d</sup> | 50<sup>d</sup> |
| 24 h after HS | 73 | 48 |
| Total leaf (4 pm) | 70<sup>d</sup> | 50<sup>d</sup> |
| Total root (4 pm) | 70<sup>d</sup> | 50<sup>d</sup> |

<sup>a</sup> Plants were heat-stressed as described by Chen et al. (31), at 38°C. 4 pm corresponds to the end of the 4-h maximum temperature stress, while 24 h corresponds to 24 h after the start of the experiment.

<sup>b</sup> Standard buffer was 10 mm Hepes, pH 8.0, 5 mm MgCl₂, 150 mm NaCl, 1 mm 2-mercaptoethanol; modifications are as indicated.

<sup>c</sup> Percent in fractions (corresponding to indicated % sucrose) as determined by counting the ¹²⁵I-labeled bands excised from Western blots except where indicated.

<sup>d</sup> Visually estimated from autoradiograms.

### Fig. 3

The 200- and 230-kDa complexes containing PsHSP21 remain intact on non-denaturing gels following chloroplast lysis under different buffer conditions. Chloroplast extracts were prepared as described under "Materials and Methods" in a standard buffer containing 10 mm Hepes, pH 8.0, 5 mm MgCl₂, 150 mm NaCl, 1.0 mm 2-mercaptoethanol and protease inhibitors, or in a modified standard buffer as indicated. Lane 1, standard buffer (Std); lane 2, minus NaCl, lane 3, with 500 mm NaCl; lane 4, with 500 mm NaCl and 20 mm MgCl₂; lane 5, minus NaCl, plus 150 mm KCl; lane 6, plus 20 mm DTT; lane 7, plus 10 mm Mg ATP; lane 8, plus 1.0% Triton X-100; lane 9, plus 2.0% Triton X-100; lane 10, minus MgCl₂, plus 0.75 mm EDTA. Following electrophoresis gels were blotted to nitrocellulose and reacted with PsHSP21 antibodies. Antibodies were detected using chemiluminescence. Positions of the 230- and 200-kDa forms of PsHSP21 are indicated at the right.

### Import Kinetics and Protease Sensitivity of PsHSP21 Transported into Isolated Chloroplasts—

To study further the high molecular weight PsHSP21-containing complexes, experiments were designed to examine the size of PsHSP21 following import into isolated chloroplasts. Assembly of chloroplast-soluble and membrane protein complexes has been shown to proceed faithfully in an isolated chloroplast system (37, 38). Following the assembly of PsHSP21 into high molecular complexes in such a system was therefore undertaken to provide independent confirmation of the native size of PsHSP21 and to define conditions required for assembly of the PsHSP21-containing complexes. It was of particular interest to determine if factors produced during heat stress were required for PsHSP21 assembly, as tested by examining PsHSP21 assembly in chloroplasts isolated from control or heat-stressed plants.

In vitro translated, [³⁵S]Met-labeled PsHSP21 precursor was mixed with isolated chloroplasts, and import and processing of PsHSP21 was examined by SDS-PAGE of the soluble protein fraction from the reisolated intact chloroplasts. Import either
was allowed to proceed continuously for up to 23 min or, to facilitate assessment of assembly, was terminated after 3 min by the addition of nigericin followed by an additional post-import incubation period of 3–20 min (samples 2, 4, 6, and 8) (+nig). After post-import incubation and before reisolation, chloroplasts were either left untreated (samples 1, 2, 5, and 6) (+therm) or were incubated with the protease thermolysin at 100 μg/ml for 15 min at room temperature in the light (+therm). Recovery of PsHSP21 was examined by SDS-PAGE and fluorography and quantified by determining the radioactivity in PsHSP21 bands on the gel. The graphs show the mean amount of PsHSP21 from at least two separate experiments (bar, S.E.), except that sample 7 was from a single experiment. The lower panel shows segments of representative gels from the different treatments as indicated.

The amount of PsHSP21 recovered over time during the import experiment is shown in Fig. 4. When PsHSP21 was imported into control chloroplasts without adding nigericin to block import and without treating with protease, the amount of PsHSP21 imported into chloroplasts continued to increase throughout the 23-min incubation, with a maximal rate in the first 10 min. When the ionophore nigericin was added to the import reaction after 3 min, no increase in imported PsHSP21 was observed between 3 and 23 min. Therefore, nigericin effectively uncoupled chloroplast photophosphorylation (33) and terminated the import. When the protease thermolysin was used to treat control chloroplasts, the same level of PsHSP21 was recovered from thermolysin-treated and untreated chloroplasts (Fig. 4A). This is consistent with the previous observation that PsHSP21 is well protected from protease digestion after import into chloroplasts from control plants (4, 27). Thermolysin treatment also effectively blocked further import; samples treated with and without thermolysin showed the same total imported cpm, although incubation in the light was continued during the thermolysin treatment. Presumably the protease immediately degrades the available precursor and thereby terminates import.

For experiments with heat-stressed chloroplasts, chloroplasts were isolated from plants 18 h following the end of the stress treatment because sufficient yields of intact organelles for import experiments were difficult to obtain directly following the stress. Our previous studies have shown that chloroplast PsHSP21 is present at about 82% of the maximum level at this time (32). Results showed that PsHSP21 import kinetics were similar, but not identical, to that of the control chloroplasts (Fig. 4B). Within 20 min the total amount of PsHSP21 imported into chloroplasts from control or heat-stressed plants was similar, constituting 18–20% of the total PsHSP21 used in the import reaction. As shown for control chloroplasts, nigericin effectively blocked import of proteins into chloroplasts from heat-stressed plants. However, in contrast to control chloroplasts, protease treatment had a dramatic effect on the recovery of PsHSP21 from chloroplasts of heat-stressed plants. As shown in Fig. 4B, only about half as much PsHSP21 was recovered from the thermolysin-treated chloroplasts compared to the untreated chloroplasts in both continuous and nigericin-terminated import experiments. In the experiment with nigericin, the amount of PsHSP21 recovered from thermolysin-treated chloroplasts increased during the first 10 min of post-import incubation. There was no evidence that the thermolysin treatment resulted in general proteolysis of many chloroplast proteins as assessed on Coomassie Blue-stained gels (not shown).

Assembly of PsHSP21 following Import into Isolated Chloroplasts—In order to determine if imported PsHSP21 was found in the high molecular weight forms in isolated chloroplasts, samples from imported treated with nigericin were analyzed on sucrose gradients. The distribution of PsHSP21 on the gradients was visualized by SDS-PAGE and fluorography, and quantified by measuring the radioactivity in PsHSP21 bands on the gel (Fig. 5). When import was performed using chloroplasts isolated from control plants and the chloroplasts were lysed immediately after the 3-min incubation (no post-import incubation) the majority of PsHSP21 (approximately 69%) was recovered in fractions 2 and 3 (19–21% sucrose) sedimenting at about 5 S (Fig. 4A). A similar sedimentation pattern was also observed during the subsequent 20-min incubation period (Fig. 5A). Therefore, throughout the import and post-import period, the majority (>60%) of PsHSP21 was recovered in a 5 S form, and less than 10% of PsHSP21 was detected in fractions 5 or 6 corresponding to the 10–11 S form seen in the in vivo heat shock extracts. Protease treatment of control chloroplasts did
not affect the recovery or distribution of PsHSP21 in the gradient (Fig. 4A).

In import experiments using chloroplasts from heat-stressed plants, the sedimentation pattern of PsHSP21 was very different from that seen in control chloroplasts (Fig. 5B). Similar to control chloroplasts, when no post-import incubation was performed the majority (approximately 65%) of PsHSP21 was recovered in fractions 2 and 3 (5 S). However, after 20 min of post-import incubation, the labeled PsHSP21 sedimented in two main peaks, one in the 5 S region (fractions 2 and 3), and another new peak in the 10–11 S region (fractions 5 and 6) (Fig. 5B). This new high molecular weight peak of imported protein cofractionated with the endogenous PsHSP21 as determined by Western blot analysis, indicating that the 10–11 S form of the imported PsHSP21 was correctly assembled (not shown). Quantitative analysis of the distribution of PsHSP21 in the gradients revealed that at least 50% of imported PsHSP21 assembled into the 10–11 S form within 20 min following import (Fig. 5B).

When chloroplasts from heat-stressed plants were treated with thermolysin, the distribution of PsHSP21 recovered from the gradients was changed significantly. As shown in Fig. 5B, the 5 S form of PsHSP21 was digested by thermolysin, while the 10–11 S form was not affected by the protease treatment. Quantitative analyses suggest that digestion of the 5 S form of PsHSP21 (about 54% of total imported PsHSP21) would account for the decreased recovery of protein observed in the thermolysin-treated chloroplasts from heat-stressed plants. Increased assembly of the thermolysin-resistant 10–11 S form would also account for increased HSP21 recovery with time, as seen in Fig. 5B (sample 8).

The non-denaturing gel data indicate that the 10–11 S form of the protein comprises two separate high molecular weight species that are not resolved by sucrose gradient analysis. To determine if both of these species are present during in vitro import, the 3-min import sample and the 20-min post-import incubation sample from experiments using control or heat-stressed chloroplasts were examined by native gel electrophoresis and autoradiography (Fig. 6). In the 3-min sample, PsHSP21 imported into control chloroplasts is resolved as a low molecular weight species with an apparent mass of 42 kDa. The amount of this species remains unchanged after a subsequent 20-min incubation period, with no accumulation of high molecular weight forms. In contrast, in samples from imports using heat-stressed chloroplasts, after 3 min the 42-kDa species and both the 230- and 200-kDa species are recovered from the chloroplasts. Betascope analysis indicates that approximately 60–70% of the total PsHSP21 is in the 42-kDa form at this time. After an additional 20 min, a larger proportion of the imported protein appears in the higher molecular weight forms, with 30–40% remaining in the 42-kDa form. These data confirm that during the in organelle reaction, PsHSP21 assembles into the same native structure detected in vivo.

**DISCUSSION**

Results of this study demonstrate that, in its native state, the chloroplast smHSP is a component of high molecular mass particles. Under a variety of chloroplast lysis conditions
PsHSP21 exhibited the same sedimentation behavior on sucrose gradients and was resolved as the same two molecular mass forms of 200 and 230 kDa by non-denaturing gel electrophoresis. The integrity of the PsHSP21-containing particles in the presence and absence of salt or divalent cations, as well as detergent, suggests that strong ionic interactions and hydrophobic forces stabilize these higher order structures. Particle size was also unaltered by the addition of ATP, indicating there is no energy dependent change in particle conformation or composition. The observation of the same high molecular weight forms in total root extracts demonstrates that formation of the particles does not require photosynthetic functions but occurs in all types of plastids.

The presence of chloroplast PsHSP21 in a higher order complex is consistent with structural studies of cytoplasmic smHSPs and α-crystallins proteins which share a conserved carboxyl-terminal domain with PsHSP21 (39, 40). We hypothesize that the PsHSP21 particles we have characterized are functionally equivalent to previously characterized smHSP particles. A majority of studies have reported that the cytoplasmic smHSPs and α-crystallins are found in complexes of approximately 500 kDa. Although we have never observed PsHSP21 in discrete soluble particles larger than 230 kDa, we cannot rule out the possibility that the 200- and 230-kDa complexes are stable forms of a partially dissociated larger species. Furthermore, the methods we have used to estimate protein mass, density gradient analysis and non-denaturing gel electrophoresis, are both based on comparisons to globular protein standards. If the PsHSP21-containing particles deviate from a globular structure then their actual mass may be significantly different (41, 42).

The composition of the cytoplasmic smHSP complexes is not well characterized. The complexes are generally thought to be oligomeric structures of the HSPs themselves, as is the case for the α-crystallin complexes (43). However, the smHSP complexes have been purified to biochemical homogeneity only from HeLa cells, and in this case a high molecular weight polypeptide copurified with the smHSP (6). Although recombinant murine HSP25 formed a high molecular weight structure (7), this complex has not been directly compared to the native protein. We do not know the composition of the 200- and 230-kDa particles in the chloroplast, other than that they contain PsHSP21. Further work is needed to purify these complexes; however, in the absence of a defined assay for smHSP function, it is not yet possible to determine the structure of the active form.

In the in vitro import experiments PsHSP21 assembly is time-dependent and appears to lack a requirement for ATP. The application of the ionophore nigericin to the import reaction after 3 min did not abolish particle formation. These data indicate that the assembly process is not dependent on the presence of a proton potential across the thylakoid membrane after 3 min of import. The assembly of the PsHSP21-containing particles in the absence of high ATP in the chloroplast also suggests that chaperonin 60 (the Rubisco-binding protein or GroEL) is probably not involved in the assembly of PsHSP21-containing particles. This is consistent with the observation that no PsHSP21 was found at the position of chaperonin 60 on the sucrose gradients or non-denaturing gels, as is the case for other proteins that interact with chaperonin 60 (44).

The inability of PsHSP21 to assemble into the high molecular weight complexes in control chloroplasts could be explained in several ways. One possibility is that in chloroplasts from heat-stressed plants a pre-existing pool of unassembled PsHSP21 molecules could accelerate the kinetics of particle assembly. Because there is no PsHSP21 in control chloroplasts (32), assembly in control chloroplasts would be mass-limited, because the level of in vitro imported PsHSP21 is extremely low. Another implication of pre-existing PsHSP21 in chloroplasts from heat-stressed plants is that the appearance of in vitro imported PsHSP21 in the assembled particle could result from monomer exchange between imported and endogenous PsHSP21 molecules. Failure to assemble in control chloroplasts could also be due to the need for other heat-induced factors or to posttranslational modifications specific to the heat-stressed chloroplasts. Osteryoung et al. (45) have observed that when the chloroplast smHSP from Arabidopsis thaliana, AtHSP21, is constitutively overexpressed in Arabidopsis plants it will assemble into the native, higher order structure even in plants which have never experienced heat stress. This result suggests that additional heat-induced factors are not required for PsHSP21 assembly. However, it is possible that overexpression of AtHSP21 causes other alterations in the chloroplast that then favor assembly. We currently favor the hypothesis that assembly in control chloroplasts is mass limited. To test this hypothesis, we are developing vectors for overexpression of the PsHSP21 precursor in Escherichia coli to enable isolation of large quantities of precursor which can then be used to increase the mass of PsHSP21 imported into control chloroplasts.

In isolated chloroplasts, about 50–70% of the total imported PsHSP21 assembled into the 200- and 230-kDa forms and the rest remained in a form at approximately 5 S on sucrose gradients and 42 kDa on non-denaturing gels. In nigericin-treated chloroplasts the 5 S/42-kDa form behaved as a precursor to the 200- and 230-kDa forms; the 5 S/42-kDa form decreased concomitant with the increase in the higher molecular weight forms. Whether the small form is a dimer of PsHSP21 that is an assembly intermediate in vivo remains to be tested. The protease sensitivity of the 5 S form in chloroplasts from heat-stressed plants that otherwise appear intact is curious. We suggest this results from a heat-induced alteration of the chloroplast envelope that allows penetration by thermolysin. However, previous studies have indicated that the chloroplast envelope is quite heat-stable in vitro (46), and the protein import functions of the membranes are clearly intact. An alternative explanation is that CaCl₂ added with the thermolysin activates an endogenous chloroplast protease that accumulates or is activated following heat stress. We have not tested this possibility, nor are we aware that such a protease activity is found in chloroplasts.

Recent data suggest that the α-crystallins and smHSPs are molecular chaperones that act in an ATP-independent fashion to prevent aggregation of other proteins (25). Current models propose that they function by binding denatured substrates. Current models propose that they function by binding denatured substrates, thereby reducing their concentration and allowing free molecules to undergo folding. There is no evidence that they actively alter protein conformation as do the chaperonin 60s (47). The presence of smHSPs in three plant cell compartments, cytoplasm, endoplasmic reticulum, and chloroplast, points to a critical role for these proteins in plant stress and much additional work is required to clarify their mechanism of action and to identify their critical in vivo substrates.

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REFERENCES

1. Lindquist, S., and Craig, E. A. (1988) Annu. Rev. Genet. 22, 631–677
2. Vierling, E. (1991) Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 579–620
3. Helm, K. W., Lafayette, P. R., Nagao, R. T., Key, J. L., and Vierling, E. (1993) Mol. Cell. Biol. 13, 238–247
4. Vierling, E., Nagao, R. T., DeRoche, A. E., and Harris, L. M. (1988) EMBO J. 7, 575–581
5. Ingolia, T. D., and Craig, E. A. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2390–2394
6. Arrigo, A.-P., and Welch, W. J. (1987) J. Biol. Chem. 262, 15359–15369
Chloroplast HSP Native Structure

7. Behlke, J., Lutsch, G., Gaestel, M., and Bielka, H. (1991) FEBS Lett. 288, 119–122
8. Bentley, N. J., Fitch, I. T., and Tuite, M. F. (1992) Year 8, 95–106
9. Arrigo, A. P., and Pauli, D. (1998) Exp. Cell Res. 175, 169–183
10. Arrigo, A. P. (1998) Dev. Biol. 122, 39–48
11. Nover, L., Scharf, K.-D., and Neumann, D. (1989) Mol. Cell. Biol. 9, 1208–1308
12. Helm, K. W., LaPeyrere, P. R., Nagao, R. T., Key, J. L., and Vierling, E. (1993) Mol. Cell. Biol. 13, 235–247
13. Collier, N. C., Heuser, J., Levy, M. A., and Schlesinger, M. J. (1988) J. Cell Biol. 106, 1131–1139
14. Walsh, M. T., Sen, A. C., and Chakrabarti, B. (1991) J. Biol. Chem. 266, 20079–20084
15. Zantema, A., Verlaan-De Vries, M., Massdam, D., Bol, S., and Van der Eb, A. (1992) J. Biol. Chem. 267, 12936–12941
16. Huot, J., Roy, G., Lambert, H., Chretien, P., and Landry, J. (1991) Cancer Res. 51, 5245–5252
17. Landry, J., Chretien, P., Lambert, H., Hickey, E., and Weber, L. A. (1989) J. Cell Biol. 109, 7–15
18. Rollet, E., Laviole, J. N., Landry, J., and Tanguay, R. M. (1992) Biochem. Biophys. Res. Commun. 185, 116–120
19. Susek, R. E., and Lindquist, S. L. (1989) Mol. Cell. Biol. 9, 5265–5271
20. Petko, L., and Lindquist, S. (1995) Cell 45, 885–894
21. Gething, M. J., and Sambrook, J. (1992) Nature 355, 33–45
22. Jakob, U., Gaestel, M., Engel, K., and Buchner, J. (1993) J. Biol. Chem. 268, 1517–1520
23. Merck, K. B., Groenen, P. J. T. A., Voert, C. E. M., De Haard-Hoekman, W. A., Horwitz, J., Bloemendal, H., and De Jong, W. W. (1993) J. Biol. Chem. 268, 1046–1052
24. Horwitz, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10449–10453
25. Jasnicke, R., and Creighton, T. E. (1993) Curr. Biol. 3, 234–235
26. Nieto-Sotelo, J., Vierling, E., and Ho, T.-H. D. (1990) Plant Physiol. 93, 1321–1328
27. Vierling, E., Mishkind, M. L., Schmidt, G. W., and Key, J. L. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 361–365
28. Klopstech, K., Meyer, G., Schuster, G., and Ohad, I. (1985) EMBO J. 4, 1901–1909
29. Suzs, K., and Yordanov, I. T. (1986) Plant Physiol. 81, 192–199
30. Weng, J., Wang, Z.-F., and Nguyen, H. T. (1991) Plant Mol. Biol. 17, 255–258
31. Chen, Q., and Vierling, E. (1991) Mol. Gen. Genet. 229, 425–431
32. Chen, Q., Lauzon, L. M., DeBohner, A. E., and Vierling, E. (1996) J. Cell Biol. 110, 1973–1983
33. Shavit, N., and San Pietro, A. (1967) Biochem. Biophys. Res. Commun. 28, 277–283
34. Laemmli, U. K. (1970) Nature 227, 680–685
35. Andersson, L.-O., Berg, H., and Mikaelsson, M. (1972) FEBS Lett. 20, 199–202
36. Vierling, E., Harris, L. M., and Chen, Q. (1989) Mol. Cell. Biol. 9, 461–464
37. Li, H., Theg, S. M., Bauerle, C. M., and Keegstra, K. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6748–6752
38. Roy, H., Bloom, M., Milos, P., and Monroe, M. (1983) J. Cell Biol. 94, 29–27
39. Plesofsky-Vig, N., Vig, J., and Brambl, R. (1992) J. Mol. Biol. 225, 537–645
40. de Jong, W. W., Leunissen, J. A., and Voorter, C. E. (1993) Mol. Biol. Evol. 10, 103–126
41. Martin, R. G., and Ames, B. N. (1961) J. Biol. Chem. 236, 1372–1379
42. Westwood, J. T., and Wu, C. (1993) Mol. Cell. Biol. 13, 3431–3446
43. Wistow, G., and Patigorzky, J. (1988) Annu. Rev. Biochem. 57, 479–504
44. Lubben, T. H., Donaldson, G. K., Vitanen, P. V., and Gatenby, A. A. (1989) Plant Cell 1, 1223–1230
45. Osteryoung, K. W., Pipes, B., Wehmeyer, N., and Vierling, E. (1994) NATO ASI Ser. H Cell Biol., in press
46. Krause, G. H., and Santarcus, K. A. (1975) Planta 137, 285–299
47. Lorimer, G. H., Todd, M. J., and Vitanen, P. V. (1993) Phils. Trans. R. Soc. Lond. Biol. Sci. 339, 297–304