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Identification of heat shock protein hsp70 homologues in chloroplasts

(envelope membrane/protein transport)

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ABSTRACT **Cytoplasmic members of the heat shock protein hsp70 family have recently been implicated in the transport of proteins to the endoplasmic reticulum and mitochondria. In addition, other hsp70 homologues have been found in the endoplasmic reticulum and mitochondria and, at least for the endoplasmic reticulum hsp70 homologue, may be involved in the proper folding and assembly of newly transported proteins. Since chloroplasts are an important site of protein transport in plant cells, we were interested in determining whether hsp70 proteins might be located in this organelle. By using immunoblotting techniques and two antibody preparations against hsp70 proteins, we have identified three chloroplastic proteins of approximately 70 kDa that are related to hsp70 proteins. One of these proteins was tightly associated with the outer envelope membrane and was not exposed at the outer surface of the chloroplasts. The other two were soluble proteins located in the stroma. Steady-state levels of the chloroplastic hsp70 homologues did not change after heat stress nor were any additional hsp70 homologues detected in chloroplasts isolated from heat-stressed plants. We discuss the possible functions of these hsp70 homologues in the transport of proteins into and within chloroplasts.**

Heat shock proteins (hsps) and their constitutively expressed homologues are predicted to perform fundamental activities in cell metabolism because of their high degree of conservation and their presence in several different cellular compartments (1). Recent evidence points to a role of at least some hsps in protein transport and assembly processes. Members of the hsp60 family, termed chaperonins, are involved in the assembly of protein complexes in bacteria (2), chloroplasts (2), and mitochondria (3, 4). GroEL, an hsp60 of *Escherichia coli* associates transiently with newly synthesized proteins *in vitro* and may facilitate protein transport into plasma membrane vesicles (5). Work from several groups implicates members of the hsp70 family in the transport of proteins into the endoplasmic reticulum (ER) (6–8) and mitochondria (6, 9). It is proposed that cytoplasmic hsp70s facilitate transport by interacting with precursor proteins and maintaining them in a transport-competent conformation. In addition, an hsp70 homologue located within the ER may be involved in folding of newly transported proteins (10). An hsp70 is also found in yeast mitochondria (11) and could serve a similar function.

Given the apparent involvement of hsp70s in protein transport in other systems, we were interested in determining whether members of this hsp family might also be found in chloroplasts. Transport of proteins, both into and within chloroplasts, is essential for the biogenesis and functioning of this organelle. Chloroplasts have already been shown to contain small hsps (12) as well as the hsp60-related protein mentioned above (2). We report here the identification of three chloroplastic proteins that are antigenically related to hsp70s. These hsp70 homologues are located in the outer envelope membrane and stroma. They are present under normal growth conditions and show little change in steady-state levels during heat-stress treatment. These results extend the possible roles of hsp70s to functions that take place in this important organelle.

MATERIALS AND METHODS

Preparation of Chloroplastic Protein Fractions. Pea (*Pisum sativum*) plants were grown at 18°C under fluorescent light (12 hr light/12 hr dark). Intact chloroplasts were prepared as in ref. 13 and fractionated using a modification of the procedure described in ref. 14. Lysed chloroplasts were centrifuged (4500 × *g*, 15 min) to yield a thylakoid pellet and a supernatant consisting of crude envelope membranes and soluble proteins. Thylakoids were washed once in 10 mM *N*-tris(hydroxymethyl)methylglycine·KOH, pH 7.6/2 mM EDTA (TE). Envelopes were sedimented 1 hr at 150,000 × *g* in a Beckman 45 Ti rotor. The resulting crude envelope pellet was fractionated into inner and outer membranes (14). Residual envelopes were removed from the supernatant by centrifuging 2 hr at 200,000 × *g* in a Beckman SW 50.1 rotor, yielding a soluble protein fraction. Whole leaf proteins were prepared as in ref. 15.

Protease Treatments of Intact Chloroplasts and Chloroplastic Fractions. Intact chloroplasts [10 mg of chlorophyll in 5.0 ml of 50 mM Hepes, pH 7.6/330 mM sorbitol (IB)] were added to 5.0 ml of IB containing: no additions, thermolysin at 200 μg/ml (Calbiochem) plus 1.0 mM $CaCl_2$, or trypsin at 300 μg/ml (Boehringer Mannheim) plus α-chymotrypsin at 300 μg/ml (Worthington). Samples were incubated 30 min on ice (except the sample treated with trypsin plus chymotrypsin that was incubated at room temperature) with occasional gentle mixing. To terminate protease reactions, 5.0 ml of IB containing no additions, 15 mM EDTA, or 6.75 mg of soybean trypsin inhibitor was added and samples were incubated 5 min on ice. Intact chloroplasts were repurified by centrifuging through 40% (vol/vol) Percoll in IB containing 5 mM EDTA or 1 mM phenylmethylsulfonyl fluoride (trypsin plus chymotrypsin-treated sample), washed once in IB, and lysed as in ref. 14. Phenylmethylsulfonyl fluoride (1 mM) was added at all subsequent steps to samples treated with trypsin plus chymotrypsin. Mixed inner and outer envelope membranes were isolated by layering crude envelopes (see above) over 1.0 M sucrose in TE and centrifuging 1 hr at 38,000 rpm in a Beckman SW 50.1 rotor.

NaCl and Alkaline Washes of Outer Envelope Membranes. Purified outer envelope membranes were washed in 2.0 ml of TE, TE plus 1.0 M NaCl, or 100 mM Na_2CO_3 (pH 11.0) with sonication (three 10-sec bursts at 25–30 W). Washed enve-

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Abbreviations: hsp, heat shock protein; ER, endoplasmic reticulum.
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lopes were diluted to 5.0 ml with TE, TE plus 1.0 M NaCl, or 200 mM Hepes·KOH (pH 7.5) and centrifuged 1.5 hr at 38,000 rpm in a Beckman SW 50.1 rotor.

Heat-Stress Treatment. Pea plants were grown on a 16 hr/8 hr, 22°C/18°C day/night cycle, respectively, for 11 days and then either heat stressed for 4 hr at 38°C as described (15) or not stressed. Intact chloroplasts were isolated and fractionated to yield mixed inner and outer envelope membranes as above.

Antibodies and Immunoblotting. Anti-tomato hsp70 antibodies were prepared in rabbits by using tomato hsp70 purified by two-dimensional gel electrophoresis as immunogen (ref. 16; antibodies were a gift of L. Nover, Halle, G.D.R.). Anti-hsp70 peptide, directed against a highly conserved region near the NH_2 terminus of hsp70 (17) was prepared using an unconjugated 11-amino acid synthetic peptide, NH_2-Val-Gly-Ile-Asp-Leu-Gly-Thr-Thr-Tyr-Ser-Cys-COOH, as immunogen (ref. 11; serum was a gift from E. Craig, University of Wisconsin, Madison).

Antibodies specific for hsp70 were affinity purified from these sera by adsorption onto immobilized bovine hsp70 (ref. 18; gift of V. Guerriero, University of Arizona) as described in ref. 19. The bovine hsp70 preparation consisted primarily of the major constitutive cytoplasmic forms found in muscle and was essentially free of ER forms.

Antibodies against outer membrane proteins were produced in chickens using pea chloroplastic outer membranes (essentially free of inner membranes) as immunogen and were isolated from egg yolks (20).

Antibodies against mixed inner and outer envelope membrane proteins were prepared in rabbits using mixed envelope membrane proteins as immunogen.

Immunoblotting was as described in ref. 19 except proteins were transferred to Immobilon-P (Millipore) in 25 mM Tris/192 mM glycine, pH 8.3. Proteins recognized by antibodies were detected using alkaline phosphatase-conjugated secondary antibodies and nitroblue tetrazolium/bromochloroindolyl phosphate (19).

RESULTS

The presence of hsp70 homologues was examined in fractions isolated from intact pea chloroplasts. The chloroplasts, isolated using Percoll density gradients, were essentially free of contamination by other organelles (21). Proteins in the chloroplastic fractions were analyzed for hsp70 homologues by using SDS/PAGE and immunoblotting techniques. Immunoblots were probed with two preparations of antibodies against hsp70s: anti-tomato hsp70 and anti-hsp70 peptide (directed against a highly conserved region of hsp70s). To reduce nonspecific protein detection, antibodies specific to hsp70 were purified from the above preparations by using bovine hsp70 as an affinity matrix.

hsp70 Homologue Associated with the Chlorosplastic Outer Envelope Membrane. A 75-kDa protein was detected in the chloroplastic envelope membranes when immunoblots were probed with either of the antibody preparations (Fig. 1 *A* and *B*). This 75-kDa protein was most abundant in the outer membrane fraction; however, some was also detected in the inner membrane fraction. Its presence in both envelope fractions can be explained by contamination of the inner membrane fraction by proteins of the outer membrane since it is currently not possible to obtain inner membranes that are free of outer membranes (Fig. 1*C* and ref. 21). The distribution of the 75-kDa hsp70 homologue of the chloroplastic envelope is, therefore, what would be expected of a protein associated with the outer membrane.

A major protein band with an apparent molecular mass of 75 kDa was seen when outer envelope membrane proteins from pea chloroplasts were separated by SDS/PAGE and stained with Coomassie blue (Fig. 1*C*). We believe that this major protein is distinct from the 75-kDa hsp70 homologue because several antibody preparations that reacted strongly with the major 75-kDa envelope membrane protein failed to recognize purified bovine hsp70 on immunoblots (J.S.M., unpublished data). This indicates that the major 75-kDa outer membrane protein is not homologous to hsp70s.

Interestingly, the two hsp70 antibody preparations used showed different reactivity patterns both with purified hsp70s and plant protein fractions. Anti-tomato hsp70 appeared to be somewhat more specific than anti-hsp70 peptide because it only reacted with a subset of the hsp70 homologues that were detected by anti-hsp70 peptide. The broader specificity of anti-hsp70 peptide was not unexpected since these antibodies were generated against a highly conserved region of hsp70s.

The possibility that the 75-kDa hsp70 homologue was a cytoplasmic contaminant bound to the surface of the chloroplasts was tested using protease treatment of intact chloroplasts. Thermolysin [a nonspecific protease that does not penetrate the chloroplastic outer membrane (22)] was used to degrade proteins exposed at the chloroplastic surface. When intact chloroplasts were treated with thermolysin, the 75-kDa hsp70 homologue associated with the outer membrane was unaffected (Fig. 2*A*), ruling out the possibility that this

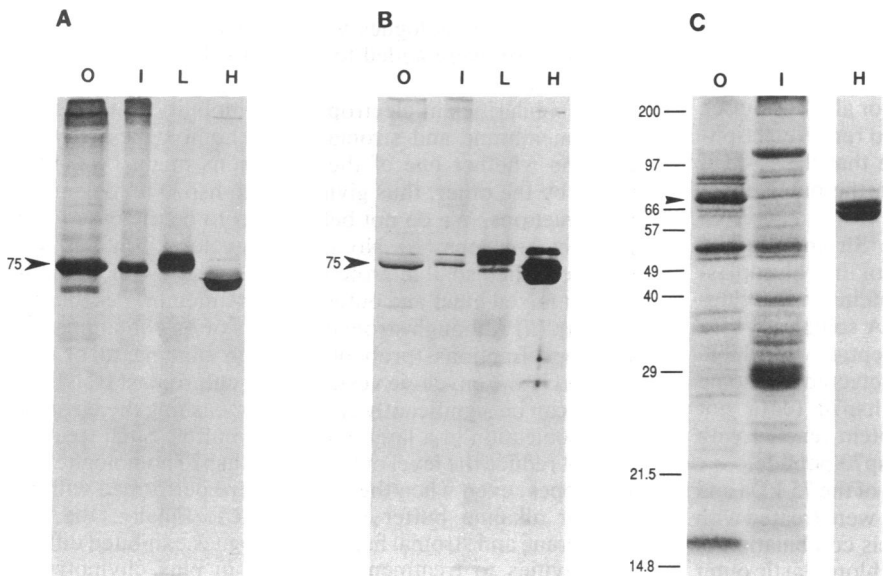

FIG. 1. Immunodetection of hsp70 homologues in chloroplastic envelope fractions. Purified outer envelope membrane proteins (lanes O), inner envelope membrane proteins (lanes I), whole leaf proteins (lanes L), and bovine hsp70 (lanes H) were separated by SDS/PAGE, transferred to Immobilon-P membranes, and probed with antibodies against hsp70s. (*A*) Immunoblot probed with affinity-purified anti-tomato hsp70. (*B*) Immunoblot probed with affinity-purified anti-hsp70 peptide. Arrowheads in *A* and *B* indicate the major 75-kDa protein detected in envelope fractions. (*C*) SDS/polyacrylamide gel stained with Coomassie blue. Arrowhead indicates position of major 75-kDa outer membrane protein. All lanes contained 12 μg of protein except lanes H, which contained 1.5 μg.

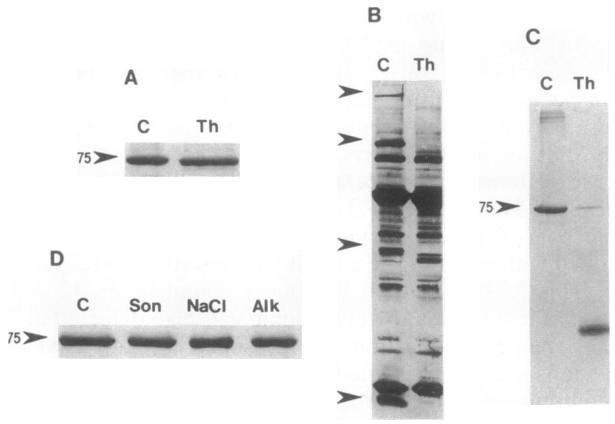

FIG. 2. Characterization of the envelope membrane association of the 75-kDa hsp70 homologue. (*A*) Immunoblot of envelope membrane proteins from control (lane C) and thermolysin-treated (lane Th) chloroplasts probed with anti-tomato hsp70. Arrowhead indicates major 75-kDa outer membrane hsp70 homologue. (*B*) Immunoblot of same samples as in *A* probed with antibodies against outer membrane proteins. Arrowheads indicate thermolysin-sensitive proteins. (*C*) Immunoblot of purified outer membrane proteins incubated in IB plus 0.1% Triton X-100 without thermolysin (lane C) or with thermolysin (lane Th), probed with anti-tomato hsp70. (*D*) Immunoblot of purified outer membrane proteins, washed in TE without sonication (lane C), in TE with sonication (lane Son), in TE plus 1.0 M NaCl (lane NaCl), or in 100 mM Na_2CO_3 (pH 11.0) (lane Alk), probed with anti-tomato hsp70. All lanes contain 10 μg of protein.

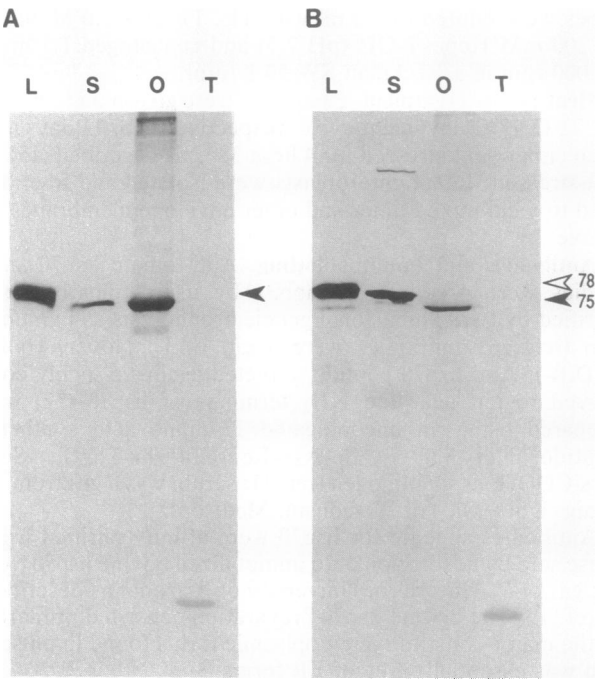

FIG. 3. Immunodetection of hsp70 homologues in various chloroplastic fractions. Immunoblots of whole leaf proteins (lanes L), chloroplastic soluble proteins (lanes S), outer envelope membrane proteins (lanes O), and thylakoid proteins (lanes T) were probed with affinity-purified anti-tomato hsp70 (*A*) or affinity-purified anti-hsp70 peptide (*B*). Solid arrowheads indicate 75-kDa proteins detected by antibodies. Open arrowhead indicates 78-kDa soluble protein detected by anti-hsp70 peptide. All lanes contain 12 μg of protein.

protein was a cytoplasmic contaminant and indicating that it probably did not have a large domain exposed at the outer surface of the chloroplasts. We verified that the protease was active by monitoring digestion of outer envelope membrane proteins known to be sensitive to thermolysin treatment (Fig. 2*B* and ref. 22). Furthermore, the 75-kDa protein was not intrinsically insensitive to thermolysin since it was degraded when isolated envelopes were treated with this protease in the presence of 0.1% Triton X-100 (Fig. 2*C*). Because the ER contains an hsp70 (1), we wanted to verify that the hsp70 homologue present in outer membrane fractions did not arise from contamination by ER. Envelope membranes do not contain glycoproteins (23), whereas many glycoproteins are found in the ER. When Con A was used to probe outer membrane proteins, no glycoproteins were detected, indicating that the outer membrane fraction was free of ER (data not shown).

We examined the nature of the envelope membrane association of the 75-kDa hsp70 homologue by subjecting isolated envelope membranes to treatments expected to remove loosely associated proteins. This protein was not removed when envelopes were sonicated in high salt or alkaline buffer (Fig. 2*D*), treatments that have been used to remove peripheral membrane proteins (24). We conclude that the 75-kDa hsp70 homologue is tightly associated with the outer envelope membrane.

hsp70 Homologues of the Chloroplastic Stroma. In the course of examining the hsp70 homologues of the chloroplastic envelope, we also observed related proteins in the chloroplastic soluble protein fraction (Fig. 3). A soluble 78-kDa protein reacted strongly with anti-hsp70 peptide. A soluble protein of the same mobility was also detected on some immunoblots probed with anti-tomato hsp70 (data not shown). In addition, a 75-kDa soluble protein reacted with anti-tomato hsp70 and weakly with anti-hsp70 peptide.

To determine more precisely the location of the 75-kDa and 78-kDa soluble proteins, intact chloroplasts were treated with a mixture of trypsin and chymotrypsin. This combination of proteases has been shown to penetrate the chloroplastic outer membrane but not the inner membrane (21); therefore, proteins of the envelope intermembrane space should be susceptible to digestion but those of the stroma should not. Neither of the soluble hsp70 homologues was digested by trypsin plus chymotrypsin in intact chloroplasts (Fig. 4*A*). This indicates that both proteins were located in the stroma and not in the intermembrane space and further verifies that neither of the soluble hsp70 homologues seen in chloroplasts was a contaminating cytoplasmic protein. In contrast, the 75-kDa hsp70 homologue associated with the outer membrane was degraded by trypsin plus chymotrypsin (Fig. 4*B*). The digestion of inner membrane proteins was monitored to verify the penetration of the outer membrane by the proteases (Fig. 4*C*). Furthermore, both the 75-kDa and 78-kDa soluble hsp70 homologues were digested when trypsin and chymotrypsin were added to isolated soluble proteins (data not shown).

The similarities in electrophoretic mobility of the 75-kDa outer membrane and stromal hsp70 homologues led us to question whether one of these fractions might be contaminated by the other, thus giving rise to hsp70 homologues in both fractions. We do not believe this to be the case for the following reasons. (*i*) No major envelope proteins were detected when soluble proteins were probed with antibodies against mixed inner and outer envelope membranes (data not shown). (*ii*) Although stromal proteins often contaminate the envelope fractions (probably through binding to or being trapped in the envelope vesicles after chloroplast lysis), their levels can be significantly reduced by washing the envelopes with sonication in a large volume of buffer. Such treatment did not reduce the level of the 75-kDa hsp70 homologue in the envelopes, even when the washes were performed with high salt or alkaline buffer (see Fig. 2*C*). Finally, the outer membrane and stromal hsp70 homologues exhibited different sensitivities to treatment with trypsin plus chymotrypsin.

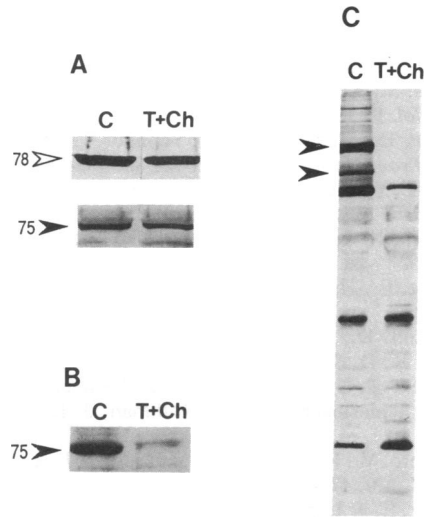

FIG. 4. Effect of trypsin plus chymotrypsin treatment of intact chloroplasts on chloroplastic hsp70 homologues. (*A*) Immunoblots of soluble proteins isolated from control (lane C) or trypsin-plus chymotrypsin-treated (lane T+Ch) chloroplasts probed with anti-hsp70 peptide (*Upper*) or with anti-tomato hsp70 (*Lower*). Both antibody preparations were used because anti-hsp70 peptide reacted most strongly with the 78-kDa soluble hsp70 homologue (see Fig. 3*A*), whereas anti-tomato hsp70 reacted most strongly with the 75-kDa hsp70 homologues (see Fig. 3*B*). Arrowheads indicate soluble chloroplastic hsp70 homologues. (*B*) Immunoblot of mixed inner and outer envelope membrane proteins from control (lane C) or trypsin-plus chymotrypsin-treated (lane T+Ch) chloroplasts probed with anti-tomato hsp70. Arrowhead indicates 75-kDa outer envelope membrane hsp70 homologue. (*C*) Immunoblot of same samples as in *B* probed with antibodies against chloroplastic envelope proteins. Arrowheads indicate trypsin- plus chymotrypsin-sensitive inner membrane proteins (see also ref. 21). All lanes contain 10 μg of protein.

This result indicates that the two proteins probably lie on opposite sides of the inner envelope membrane.

Effect of Heat Stress. Both constitutively expressed and heat-inducible hsp70 family members have been described (1). Therefore, we wanted to know if there was a change in the steady-state level of any of the chloroplastic hsp70 homologues in response to heat stress or if there were any additional heat-inducible hsp70 homologues in the chloroplast. Chloroplastic envelope membrane protein, soluble protein, and thylakoid fractions were isolated from plants that had either been heat-stressed for 4 hr at 38°C or had not experienced heat stress. By using antibody preparations to detect hsp70s, no significant difference was observed in the levels of the chloroplastic hsp70 homologues between stressed and unstressed plants, and no new chloroplastic hsp70s were detected (data not shown). However, hsp21, a chloroplastic low molecular weight hsp (12, 15), was found to accumulate in the chloroplasts from heat-stressed plants, indicating that the plants had experienced heat stress (data not shown).

DISCUSSION

We have identified three proteins in purified pea chloroplasts that are related to hsp70s. The chloroplasts used were free of contamination by other organelles (21) and protease treatments showed that the hsp70 homologues were not contaminating cytoplasmic proteins. The stromal and outer envelope membrane proteins, both 75 kDa, reacted most strongly with anti-tomato hsp70, although the 78-kDa stromal hsp70 homologue reacted most strongly with anti-hsp70 peptide and only weakly with anti-tomato hsp70. These differences of mobility and reactivity may reflect the origins of the chloroplastic proteins, their functions, or both. It is interesting to note that antibodies against the *E. coli* hsp70 homologue, the product of the DnaK gene, recognized the 78-kDa stromal hsp70 homologue but did not seem to recognize the 75-kDa proteins (J.S.M., unpublished data). The gene for the yeast mitochondrial hsp70, Ssc1p, has been sequenced and the predicted amino acid sequence of this protein was found to be more homologous to the DnaK gene product than to eukaryotic hsp70s (11). It is possible that the 78-kDa stromal hsp70 homologue is also more closely related to the DnaK gene product. These organelle proteins may have evolved from hsp70s found in the prokaryotic progenitors of mitochondria and chloroplasts.

The three chloroplastic hsp70 homologues are almost certainly encoded on nuclear genes and synthesized in the cytoplasm. Support for this conclusion comes from the nucleotide sequence of the chloroplast genome that does not contain any regions encoding a protein related to hsp70s (25). This situation is similar to that of the yeast mitochondrial hsp70 homologue, Ssc1p, which has been shown to be nuclear encoded. This protein is synthesized as a higher molecular weight precursor and is transported posttranslationally into mitochondria (11).

Levels of the chloroplastic hsp70 homologues did not appear to change after heat stress. Furthermore, no new hsp70 homologues were detected in chloroplasts after heat treatment. This is in dramatic contrast to the synthesis of the low molecular weight chloroplastic hsp that is undetectable at control temperatures but increases to high levels during heat stress (15). Effects of heat treatment on hsp70s might not have been detected by the methods we used since, at least in *Drosophila*, levels of heat-induced hsp70s are less than or equal to levels of constitutively produced hsp70 homologues (26). Changes in the amounts of hsp70 homologue mRNA or in the rate of protein synthesis or turnover might not be reflected in the steady-state protein levels. A more sensitive assay of the heat inducibility of these proteins would be to examine the levels of mRNA for each protein present before and after heat treatment.

Although nothing is known concerning the roles of hsp70 homologues in chloroplasts, it is likely that these proteins have functions similar to those of hsp70s found in other cellular compartments. Recent work has suggested (6–9) that hsp70s are involved in protein transport to mitochondria and the ER. It is thought that these proteins use the energy of ATP hydrolysis to maintain precursor proteins in a transport competent conformation in the cytoplasm. Immunoglobulin heavy chain binding protein (BiP), an hsp70 homologue of the ER lumen, is known to associate with aberrant proteins (10) and may serve to unfold and/or refold such proteins. The *E. coli* hsp70 homologue, the DnaK gene product, is known to be necessary for phage λ DNA replication (27). Its role in replication was proposed to be facilitating ATP-dependent protein unfolding and disassembly during the initiation of replication (28). There are many chloroplastic processes in which the putative unfolding or assembly/disassembly functions of hsp70 homologues could be required. They may facilitate folding of newly transported proteins or may unfold misfolded proteins in the stroma. They may also be involved in transport of proteins from the stroma to other chloroplastic compartments (e.g., thylakoid membrane and lumen). Proteins transported from the stroma, whether synthesized on chloroplastic ribosomes or as intermediates in transit from the cytoplasm, may require a particular conformation for transport. Stromal hsp70 homologues could help maintain these proteins in such a transport competent conformation.

It is interesting to speculate on the possible role of the outer membrane hsp70 homologue. Our protease-sensitivity data (Figs. 2 and 4) indicate that this protein is exposed at the inner

surface of the outer membrane although we cannot exclude the possibility that it is also exposed on the outer surface. It has been suggested that the site of utilization of ATP required for the binding of precursor proteins to chloroplasts is in the intermembrane space (29) and it is conceivable that binding involves hsp70 homologues. Flynn *et al.* (30) demonstrated that two hsp70 homologues, immunoglobulin heavy-chain binding protein and uncoating ATPase (hsc70), bind specific peptide sequences and release them in an ATP-dependent fashion. It is possible that the envelope hsp70 homologue is involved in the recognition of transit sequences and the binding of precursor proteins. Another possibility is that the envelope hsp70 homologue could act as a membrane bound protein unfoldase. Rothman and Kornberg (31) speculated that such unfolding enzymes might exist and could function as protein translocators, threading precursor proteins across the membrane. The next challenge will be to determine what role chloroplastic hsp70 homologues play in protein transport or other processes.

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