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A Normal Mitochondrial Protein is Selectively Synthesized and Accumulated during Heat Shock in *Tetrahymena thermophila*

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We have identified and purified a 58-kilodalton protein of *Tetrahymena thermophila* whose synthesis during heat shock parallels that of the major heat shock proteins. This protein, hsp58, was found in both non-heat-shocked as well as heat-shocked cells; however, its concentration in the cell increased approximately two- to threefold during heat shock. The majority of hsp58 in both non-heat-shocked and heat-shocked cells was found by both cell fractionation studies and immunocytochemical techniques to be mitochondrially associated. During heat shock, the additional hsp58 was found to selectively accumulate in mitochondria. Nondenatured hsp58 released from mitochondria of non-heat-shocked or heat-shocked cells sedimented in sucrose gradients as a 20S to 25S complex. We suggest that this protein may play a role in mitochondria analogous to the role the major heat shock proteins play in the nucleus and cytosol.

Virtually every organism tested to date responds to hyperthermal stress by transiently inducing the synthesis and accumulation of a specific array of polypeptides commonly referred to as heat shock proteins (hsps) or stress proteins (summarized in references 4, 32, and 48). The major hsps of most organisms fall into size classes of approximately 80 to 90, 68 to 75, and 15 to 30 kilodaltons (kDa). Evolutionary conservation of DNA and amino acid sequences exhibited by the groups of major hsps, especially the hsp70 group (6, 10, 24–26, 32, 47), suggest that they must play fundamental metabolic roles in the cell. In many organisms, a variable array of minor hsps are also synthesized during heat shock (17, 33, 38, 58, 60). Collectively, these hsps are thought to protect the cell from the adverse effects of heat shock as well as other physiological stresses (summarized in references 4, 10, 32, and 48).

While mutations in specific heat shock genes have been shown to affect the viability of the cells harboring those mutations (11, 35, 62), the exact functions of individual hsps are still not well understood. One approach to ascertaining the functions of individual hsps has been to determine the subcellular locations of those proteins. These studies have revealed that during heat shock certain hsps, such as some members of the 70-kDa group, and several of the small hsps tend to accumulate in the nucleus, while others, such as members of the 80- to 90-kDa group, remain cytosolic (3, 17, 35, 51, 56, 59). Additionally, several of the small hsps appear to form cytoplasmic aggregates (2, 40, 49). In plants, several hsps were found to associate with chloroplasts (27, 57), ribosomes (31), and mitochondria (31, 50). Although these studies clearly show differences in the localizations of the hsps, particular functions have not yet been ascribed to these proteins.

In *Tetrahymena thermophila*, the heat shock response is typical and can be induced by shifting the organism from 30 to 41°C (16, 19, 20, 63). This induces the immediate and transient synthesis of the major hsps (73 kDa, 80 kDa, and 29 to 35 kDa) as well as several minor hsps (20, 37).

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As we are interested in determining the functions of individual hsps, we have begun purifying several of these proteins so as to better analyze their metabolism and localization. We have purified one of the minor hsps, hsp58, and found that although the synthesis and accumulation of this protein is induced during heat shock in a manner parallel to that shown by the major hsps, hsp58 is also a normal component of the non-heat-shocked cell. hsp58 appears to be a normal component of the mitochondria of *T. thermophila*, and its concentration selectively increases in this compartment during heat shock. hsp58 is apparently synthesized in the cytosol, imported into mitochondria, and assembled into oligomeric structures composed primarily of the hsp58 protein.

MATERIALS AND METHODS

Culture conditions. A single strain of *T. thermophila*, CU355 (II), was used in all experiments. Cultures were grown at 30°C on a gyratory shaker in 1% proteose peptone (Difco Laboratories) supplemented with 0.003% Sequestrene (Ciba-Geigy). Heat shocks were carried out at 41°C in a shaking water bath as previously described (19, 20). Only cells in early log phase (less than 100,000 cells per ml) were used in all experiments. Cells were harvested by centrifugation at 250 × *g* for 3 min at room temperature, washed by gently suspending the cell pellet in 10 mM Tris, pH 7.5, and repelleted at 250 × *g* for 3 min.

The radioactive labeling of cellular proteins was accomplished by adding [^{3}H]lysine ([4,5-^{3}H]lysine; Amersham Corp.) at 20 μCi/ml to cultures of cells. This amino acid was used for labeling proteins because it is in very low concentration in proteose peptone.

Cell numbers were determined by diluting 1-ml samples of cell suspensions with 9 ml of 10% Formalin–0.2 M NaCl (1:8, vol/vol) and counting with a Coulter counter.

Gel electrophoresis of proteins. The procedure for the one-dimensional separation of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has been described (19, 20). Two-dimensional PAGE of proteins was performed by the procedures of Guttman et al. (17). The

first dimension was a nonequalibrium pH gradient electrophoresis run for 2,400 V-h. The second dimension was a 15% SDS-polyacrylamide gel run for 800 V-h. Both one- and two-dimensional gels were stained with Coomassie brilliant blue R and destained as described previously (19, 20). Silver staining was done by the procedure of Wray et al. (64). The fluorographic procedure of Lasky and Mills (28) was used to visualize radioactively labeled proteins. Samples of total cellular protein were prepared as described previously (19). The protein concentration in samples was determined with the BioRad protein assay system.

hsp58 purification. Cells heat shocked at 41°C for 120 min were harvested, washed once with 10 mM Tris, pH 7.5, and repelleted. The cell pellet was suspended in 8 to 10 volumes of LK-buffer (0.1 M KCl, 0.01 M $MgCl_2$, 0.01 M Tris, pH 7.5) and lysed by the addition of deoxycholate and Triton X-100 to final concentrations of 1.0% and 0.5%, respectively, followed by 10 to 15 strokes in a tight-fitting Dounce homogenizer. The cell lysate was centrifuged at 15,000 × *g* for 10 min at 4°C. The supernatant was underlaid with 1/4 volume of a 15% sucrose solution made up in HK-buffer (0.6 M KCl, 0.01 M $MgCl_2$, 0.01 M Tris, pH 7.5) and centrifuged at 100,000 × *g* for 8 h at 3°C. The pellet was suspended in HK-buffer and layered onto an HK-buffered 15 to 30% sucrose gradient. The gradient was centrifuged at 23,000 rpm for 16 h at 3°C, and 2-ml fractions were collected. Proteins in each fraction were analyzed by one-dimensional PAGE and fluorography to determine which fractions were enriched in hsp58. Proteins in the appropriate fractions were then subjected to two-dimensional PAGE, the first dimension being the acid-urea gel system of Lastick and McConkey run in tubes (29). Proteins were electrophoresed toward the anode at 1 mA per tube for 18 h. Following electrophoresis, the tube gels were equilibrated in SDS buffer (0.0625 M Tris, pH 6.8, 5% β-mercaptoethanol, 10% glycerol, 2% SDS) for 1 h before being layered onto 15% SDS-polyacrylamide slab gels, which were prepared and run as described above. The second-dimension gels were lightly stained with Coomassie brilliant blue R, and the spot corresponding to hsp58 was cut from the gel, electroeluted, dialyzed against 10 mM KCl–10 mM Tris, pH 7.5, and concentrated by ultrafiltration.

Preparation of anti-hsp58 antiserum. Purified hsp58 protein, emulsified with 3 volumes of Freund complete adjuvant, was injected into a rabbit by the procedure of Vaitukaitis (55). Approximately 100 μg of purified hsp58 protein was injected in 50-μl portions intradermally at multiple sites (approximately 20 to 30) around the back of a rabbit. The procedure was repeated 6 weeks later with approximately 50 μg of purified hsp58 emulsified in Freund incomplete adjuvant. One week following the booster injection, the rabbit was bled from the marginal ear vein. The serum was processed by standard procedures (7) to purify the immunoglobulin G (IgG) fraction.

Immunoblotting. Proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell) by a modified version of the procedure of Towbin et al. (52). The transfer solution used contained 12.5 mM Tris, 96 mM glycine, 0.05% SDS, and 10% methanol. Transfers were typically carried out at 0.6 A for 2 h. The transferred membranes were then blocked for 2 h in TBS containing 3.0% bovine serum albumin and 10.0% normal goat serum. The membranes were washed briefly in TBS (0.9% [wt/vol] NaCl, 10 mM Tris chloride [pH 7.4]) incubated at 37°C overnight with the anti-hsp58 antiserum diluted 1:1,000 in TBS containing 10.0% blocking solution, and then given six 10-min washes with TBS. The membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:3,000 in TBS containing 10.0% normal goat serum for 2 h at room temperature, followed by six 10-min washes in TBS. Detection of the antigen-antibody complexes was carried out exactly as described by Hawkes et al. (22). Quantitation of hsp58 was accomplished by separating a dilution series of known cell equivalents of protein by SDS-PAGE, transferring the proteins to nitrocellulose, and probing the nitrocellulose membrane with the anti-hsp58 antiserum. The stained and still wet nitrocellulose sheets were scanned with an LKB Ultrascan laser densitometer. The area under the peaks of the chart recorder tracings corresponding to the immunoreactions were quantitated with a Hewlet Packard Digitizer. This analysis allowed us to determine the protein concentration for each sample, which yielded a colorimetric reaction proportional to the amount of protein loaded. All samples of a particular experiment, at concentrations within the linear range of the detection reaction, were then electrophoresed on the same gel, transferred to nitrocellulose, and probed with the anti-hsp58 antiserum. The immunoreactive bands were scanned and quantitated as described above.

Immunocytochemistry. Cells were prepared for immunocytochemistry as described by Wenkert and Allis (61). Approximately 900,000 cells (10 ml of culture) were pelleted at 250 × *g* for 2 min, suspended in 10 ml of 60 mM Tris, and fixed by the addition of 10 μl of fixative (saturated $HgCl_2$–100% ethanol, 2:1) to the cell suspension. The cells were incubated in the fixative at room temperature for 5 min, pelleted, washed in 100% methanol, repelleted, and suspended in 3 ml of 100% methanol. Cells were dropped onto cover slips, dried at room temperature for 30 min, and incubated in phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM $NaPO_4$, pH 7.5) for 4 to 10 h at 4°C. Cover slips were then incubated with blocking solution (PBS containing 3% bovine serum albumin and 10% normal goat serum) for 2 h at room temperature, given two 10-min washes with PBS, incubated for at least 6 h with the appropriate antiserum diluted 1:500 with PBS containing 10% blocking solution, washed six times for 10 min each in PBS, incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma) diluted 1:500 with PBS containing 10% normal goat serum for 2 h, and washed six times for 10 min each with PBS. Cover slips were then mounted on slides, and the cells were viewed in an Olympus BH-2 microscope fitted with a reflected light fluorescence attachment (model BH-2-RFL) and a BP-490 exciter filter.

Mitochondria isolation. The procedure used for isolation of mitochondria was based on that described by Morin and Cech (39). Cells were harvested by centrifugation, washed once with 10 mM Tris–1 mM EDTA, pH 7.5, and suspended in ice-cold buffer containing 0.35 M sucrose, 100 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 10 mM Tris, pH 7.5. All following steps were performed at 4°C. Cells were disrupted by two passages through a Fisher portable homogenizer, and the lysed cell suspension was centrifuged at 3,000 × *g* at 4°C for 5 min. The resulting supernatant was centrifuged two more times; each time the pellet was discarded. The final supernatant was then layered onto a linear 0.75 to 1.75 M sucrose gradient containing 100 mM EDTA and 10 mM Tris, pH 7.5, and centrifuged at 25,000 rpm in a Beckman SW28 rotor for 240 min at 4°C. The band of light-scattering material sedimenting approximately 2/3 of the way down the gradient was found to contain mitochondrial enzyme activity. In some cases, the purified mitochondrial band was collected, diluted 1:2 with buffer containing

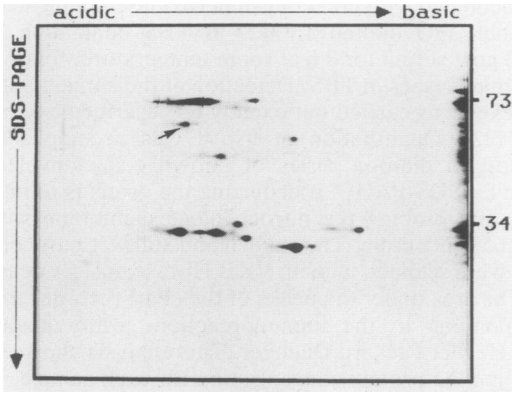

FIG. 1. Proteins synthesized by *T. thermophila* during early heat shock. Growing cells were shifted from 30 to 41°C and labeled with [^{3}H]lysine from 5 to 30 min after the initiation of heat shock. Total cell protein was separated by two-dimensional gel electrophoresis (nonequilibrium pH gradient electrophoresis followed by SDS-PAGE; see Materials and Methods), and the labeled proteins were visualized by fluorography. A portion of the sample was also loaded and run in the second-dimension gel system only, as seen along the right margin of the fluorogram. One of the major hsps, hsp80, although obvious in the one-dimensional separation, was not resolved in this two-dimensional gel system. The numbers on the right indicate molecular mass (in kilodaltons). The arrow indicates the position of hsp58.

100 mM EDTA and 10 mM Tris, pH 7.5, and then pelleted by centrifugation at 15,000 × *g* for 15 min.

Enzyme assays. Succinate ferricyanide reductase activity was assayed by the procedure of Lloyd et al. (34). The reaction mixture contained 13.3 mM sodium succinate, 10 mM KCN, 1 mM $K_2Fe(CN)_6$, and 100 mM potassium phosphate, pH 7.4. Reaction mixtures were assayed at 420 nm against control cuvettes lacking succinate. Lipoamide dehydrogenase activity was assayed by the procedure of Reed and Willms (45). The reaction mixture contained 0.3 mM β-NAD, 0.1 mM β-NADH, 3.0 mM lipoamide (6,8-thiotic acid amide), and 150 mM potassium phosphate, pH 8.1. Reaction mixtures were assayed at 340 nm against control cuvettes lacking lipoamide.

RESULTS

During the first 30 min of a 41°C heat shock of *T. thermophila*, hsp synthesis is maximally induced while non-hsp synthesis is greatly repressed (15, 17, 19, 20, 63). The hsps synthesized by *T. thermophila* during this early stage of heat shock are shown in Fig. 1. These include a minor hsp of 58 kDa, referred to here as hsp58. As with the major hsps of this organism, hsp58 has been shown to undergo a transient induction in its synthesis during the first hour of heat shock (37).

Purification of hsp58. To purify hsp58, we took advantage of one of its more interesting properties, which became manifest when we subjected heat-shocked cells to subcellular fractionation. When the 10,000 × *g* supernatant of detergent-lysed, heat-shocked cells labeled with [^{3}H]lysine during the first 30 min of the heat shock was subjected to high-speed (100,000 × *g*) centrifugation, the majority of the radioactive hsp58 was recovered and highly enriched in the crude ribosome pellet. When the pelleted material was suspended in a medium which dissociated ribosomes into subunits and then subjected to centrifugation on a sucrose gradient (Fig. 2), very little of any of the radioactive hsps,

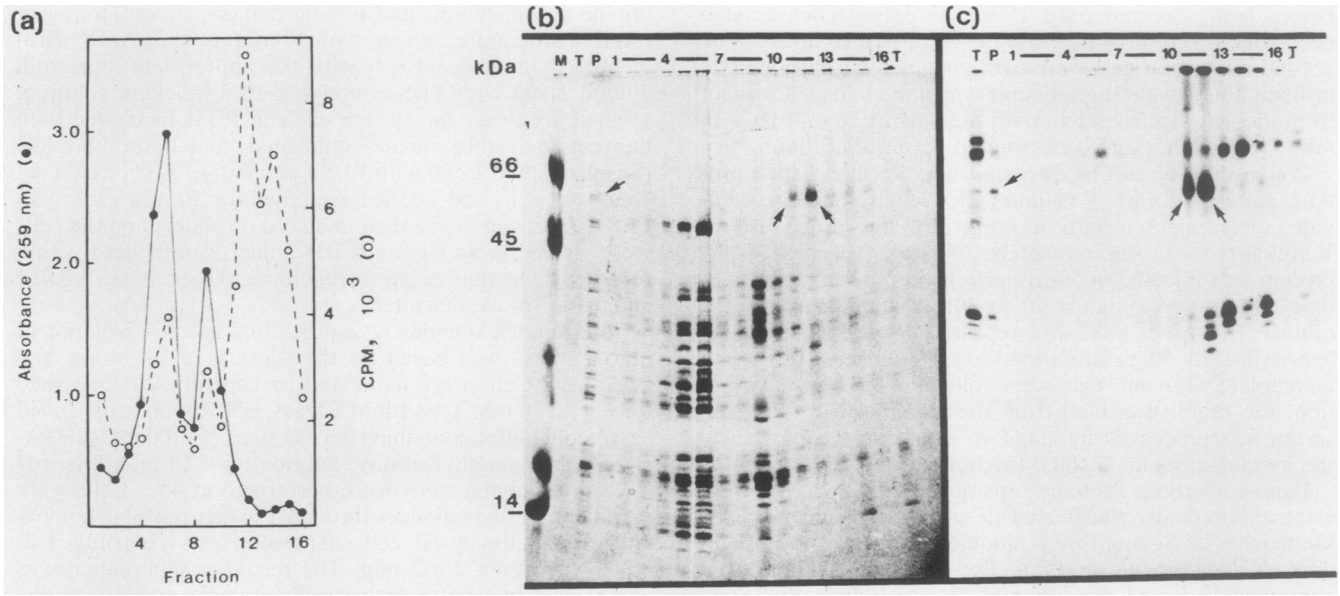

FIG. 2. hsp58 copurifies with ribosomes but is not ribosome associated. Cells were labeled with [^{3}H]lysine from 5 to 30 min of a 41°C heat shock. They were then harvested and used to prepare a total protein sample (T) and a 100,000 × *g* pellet (P), which contained the majority of the ribosomes. The 100,000 × *g* pellet was suspended, and its contents were centrifuged through a linear 15 to 30% sucrose gradient, which causes dissociation of ribosomes into ribosomal subunits (see Materials and Methods). (a) Positions of the ribosomal subunits in the gradient were determined from the absorbance of each fraction at 259 nm, and the positions of the labeled hsps were monitored by the acid-precipitable counts in each fraction. The direction of sedimentation is from right to left. (b and c) Total protein (T), proteins in the 100,000 × *g* pellet (P), and proteins in each fraction of the sucrose gradient (numbered lanes) were separated by SDS-PAGE and detected by staining with Coomassie brilliant blue R (b) and subsequently by fluorography (c). hsp58 is indicated by the arrows. Protein size standards are shown in lane M. An error in the loading of the gel resulted in the following order of sucrose gradient fractions for panels b and c: 1 to 12, 14, 15, 13, 16.

including hsp58, was found to be associated with either ribosomal subunit (Fig. 2c). Although not subunit-bound under the salt conditions employed (0.6 M KCl), hsp58 still did not exist as a free protein. Instead, it sedimented as a particle of approximately 20 to 25S. This sedimentation behavior explains why it could have pelleted with ribosomes under the conditions we used for obtaining the crude ribosome fraction. As can be seen in Fig. 2c, hsp58 sedimented to a position in the gradient (fraction 12) which allowed it to be well resolved from the ribosomal subunit proteins (Fig. 2b, fractions 5-6 and 9-10) and from many of the other contaminating nonribosomal proteins which initially pelleted with the ribosomes (fractions 14 to 16). The stained proteins in fraction 12 (Fig. 2b) included a prominent band at 58 kDa, presumably hsp58. Two separate two-dimensional gel analyses (see Materials and Methods) of the proteins found in fraction 12 showed that the major stainable protein species of that fraction coelectrophoresed with the labeled hsp58 (data not shown), verifying that this protein was hsp58.

Final purification of hsp58 was achieved by subjecting partially purified hsp58 (obtained from the appropriate fractions of sucrose gradients similar to that shown in Fig. 2) to two-dimensional gel electrophoresis, cutting the hsp58 protein spot from the gel, and electroeluting the protein from the gel. The protein obtained in this manner was estimated to be at least 95% pure (data not shown, but see lane 3 of Fig. 3a). A polyclonal anti-hsp58 antiserum was generated in a rabbit by injection of the purified hsp58 protein. By Western blot (immunoblot) analysis, the immune serum was found to react with a 58-kDa protein in a sample of total proteins from heat-shocked cells as well as with the purified hsp58 protein (Fig. 3a). Preimmune serum showed no reaction with any *T. thermophila* protein (Fig. 3a). To check whether the immune serum recognized only hsp58, total proteins from cells labeled with [^{3}H]lysine from 5 to 30 min of heat shock were separated by two-dimensional gel electrophoresis, blotted to nitrocellulose, and probed with the immune serum. As can be seen in Fig. 3c, the antiserum reacted almost entirely with a single 58-kDa protein spot. This immunoreactive spot corresponded to the radioactive hsp58 spot seen on the fluorogram of the Western blot, verifying that the antiserum was specific for hsp58.

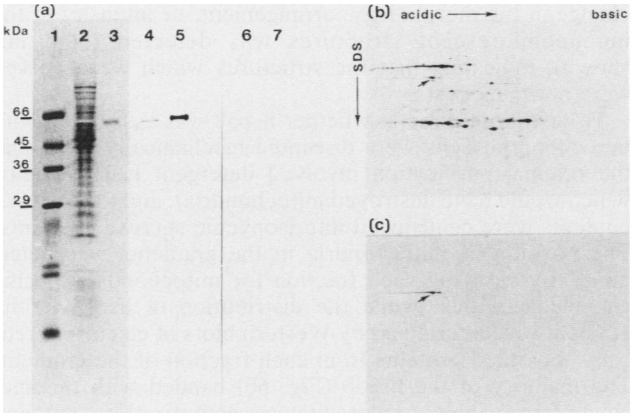

FIG. 3. Characterization of anti-hsp58 antiserum. (a) Protein samples were separated by one-dimensional SDS-PAGE and either stained with Coomassie brilliant blue (lanes 1, 2, and 3) or electrophoretically transferred to nitrocellulose membranes (lanes 4 through 7). The nitrocellulose sheets were incubated first with either anti-hsp58 antiserum (lanes 4 and 5) or preimmune serum (lanes 6 and 7) and then with peroxidase-conjugated goat anti-rabbit IgG. The immunoreactive proteins were detected as described in Materials and Methods. The samples examined were total proteins from cells heat shocked at 41°C for 60 min (lanes 2, 4, and 6) and purified hsp58 (lanes 3, 5, and 7). Protein size standards are shown in lane 1. (b and c) Total proteins from cells labeled with [^{3}H]lysine from 5 to 30 min of heat shock were separated by two-dimensional gel electrophoresis (Fig. 1), transferred to a nitrocellulose sheet, probed with the anti-hsp58 antiserum as above (c), and fluorographed (b). Arrows indicate the position of hsp58. The smearing of the proteins is atypical. However, at times, some or all of the proteins in our two-dimensional gels exhibit the streaking seen in this figure.

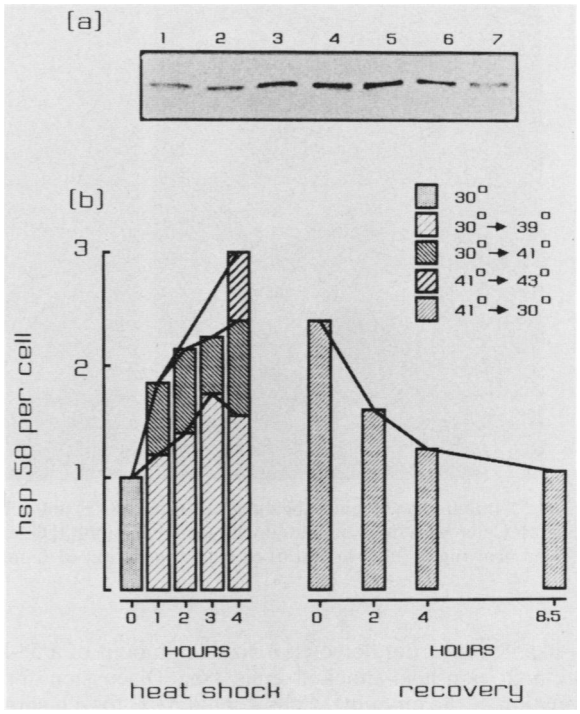

FIG. 4. Changes in cellular levels of hsp58 during heat shock and recovery. Protein samples were prepared from cells at various times following a shift from 30°C to a heat shock-inducing temperature and during recovery at 30°C from a 4-h 41°C heat shock. Heat shocks were a continuous 39°C treatment, a continuous 41°C treatment, and a 1-h 41°C treatment followed by a 3-h 43°C treatment. Recovery was at 30°C following a 4-h 41°C heat shock. Cell numbers were monitored throughout the treatments as described in Materials and Methods. Following electrophoretic separation of the total proteins from equal cell equivalents, the anti-hsp58 antiserum was used to detect the hsp58 in each cell preparation. (a) Example of the data. Samples from 30°C non-heat-shocked cells, lane 1; cells heat shocked at 41°C for 1 h, lane 2; heat shocked for 2 h, lane 3; heat shocked for 3 h, lane 4; heat shocked for 4 h, lane 5; 2 h of recovery from a 4-h 41°C heat shock, lane 6; 4 h of recovery from heat shock, lane 7. (b) To more accurately quantitate these levels, dilution series of equal cell protein equivalents of each sample were analyzed by quantitative Western blotting as described in Materials and Methods. The amounts of hsp58 per cell are in units relative to the amount of hsp58 found in non-heat-shocked cells growing at 30°C (which was assigned a value of 1). All values are averages of either two or three independent measurements. In cases of multiple components of a bar, the value of any particular component is the sum of that component and those below it.

Changes in levels of hsp58 during heat shock. To determine how heat shock affected the level of hsp58 in the cell, the antiserum was used to measure the relative cellular concentration of hsp58 throughout a continuous 41°C heat shock (Fig. 4). An unexpected outcome of this analysis was that the

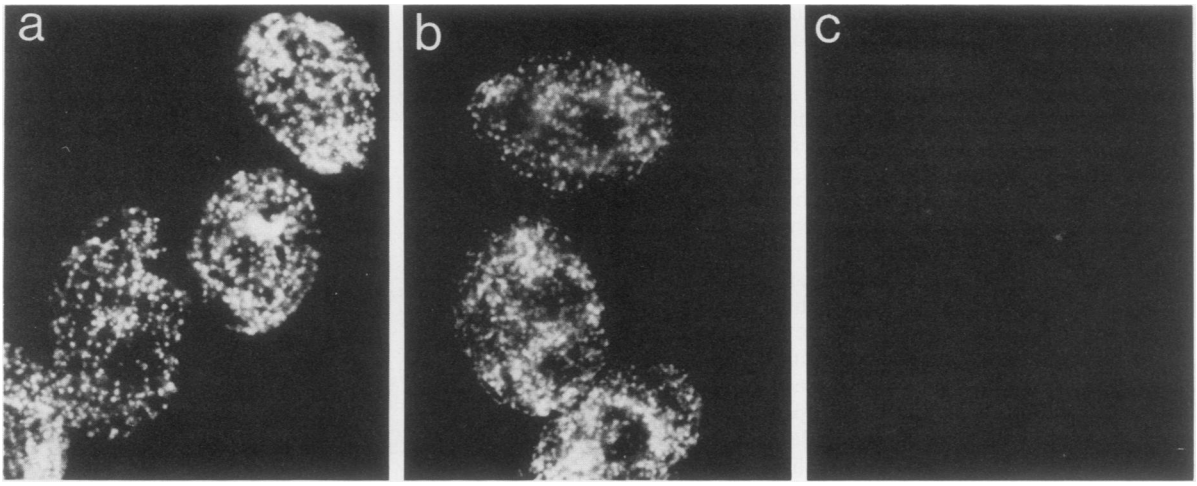

FIG. 5. Immunocytological localization of hsp58. Fixed cells were incubated with either anti-hsp58 antiserum (a and b) or preimmune serum (c). Cells were then incubated with goat anti-rabbit IgG, and the patterns of their immunofluorescence were determined. Cells examined had been growing at 30°C (a and c) or heat shocked at 41°C for 120 min (b).

anti-hsp58 antiserum detected a sizable amount of a 58-kDa protein in non-heat-shocked cells (see Discussion for an estimation of the amount). Cells kept at 41°C for 4 h showed a 2.5-fold increase in the overall cellular concentration of hsp58, with most of this increase occurring within the first 120 min of heat shock.

When cells were shifted from 41°C back to 30°C, they resumed growth and division, and the level of hsp58 gradually declined. By 510 min of recovery at 30°C, the cells had undergone two doublings and the amount of hsp58 had returned to its original non-heat-shocked level. Whether this decline was due to degradation, dilution by cell division, or both remains to be determined.

To test whether the severity of the heat shock had an effect on the extent of hsp58 accumulation, levels of this protein were measured in cells subjected to two other temperature regimens. The first was a continuous heat shock at 39°C. In this case the concentration of hsp58 increased to a maximum of 1.9-fold relative to the 30°C level. The second experiment involved shifting cells from 30 to 41°C for 1 h, followed by a shift up to 43°C for 3 h. This temperature sequence was necessary because growing cells cannot survive a direct shift from 30 to 43°C (20). In this latter experiment, the level of hsp58 increased nearly threefold relative to the 30°C level. Together with the data for the continuous 41°C heat shock, these results show that as the heat shock became more severe, the cellular concentration of hsp58 increased. A similar differential response to various heat shock temperatures has been noted for the major hsps of *Drosophila melanogaster* (33) and appears also to be the case for the major hsps of *T. thermophila* (unpublished data).

Subcellular localization of hsp58 before and after heat shock. To gain more information about hsp58 metabolism, a comparison of the intracellular distribution of this protein in non-heat-shocked and heat-shocked cells was made by indirect immunofluorescent cytological techniques. The anti-hsp58 immunofluorescence detected in non-heat-shocked cells was localized to numerous spherical structures arranged in multiple longitudinal rows along the subcortical region of the organism (Fig. 5a). Heat-shocked cells (Fig. 5b) showed similar immunofluorescent structures; however, their arrangement within the cell appeared less ordered. Although this technique is not quantitative, the immunofluorescence within the spherical structures of heat-shocked cells appeared to be more intense than that seen in the non-heat-shocked cells. The structures and their arrangement as shown in Fig. 5 appeared to be very similar to that which has been described for the mitochondria of *T. thermophila* (5, 12, 13, 23). However, mucocysts may show a similar arrangement in this organism (1). Discharge of the mucocysts can be induced by the dye alcian blue (42). When cells were treated with the dye and mucus was released, no change in the morphology, arrangement, or intensity of the immunofluorescent structures was detected (data not shown), indicating that the structures which we observed were not mucocysts.

To test more directly whether hsp58 was associated with mitochondria, cells were disrupted mechanically (note that the original purification involved detergent lysis of cells, which would have destroyed mitochondria), and the released contents were centrifuged into isopycnic sucrose gradients. The position of mitochondria in the gradients was determined by assaying each fraction for mitochondria-specific enzyme activities, while the distribution of hsp58 in the gradient was determined by Western blots of electrophoretically separated proteins from each fraction of the gradient. The majority of the hsp58 (Fig. 6b) banded with the succinate-ferricyanide oxidoreductase activity (Fig. 6a) and lipoamide dehydrogenase activity (data not shown). As qualitatively identical results were obtained for both non-heat-shocked and heat-shocked cells (data not shown), the increase in the total amount of hsp58 in the cell was apparently due to a proportional increase in the amount of hsp58 found in the mitochondrial fraction. To measure directly the amount of mitochondrial hsp58, mitochondria were purified from non-heat-shocked cells and from cells kept at 41°C for 180 min. The relative amount of hsp58 in both samples was determined by quantitative Western blot analysis of the purified mitochondrial proteins, as had been done with whole cells. This analysis (Fig. 7) revealed that the level of hsp58 within mitochondria increased approximately two to threefold during the 41°C treatment, just as was found for whole cells. The selective nature of this increase can even be seen in the increased staining intensity of a 58-kDa

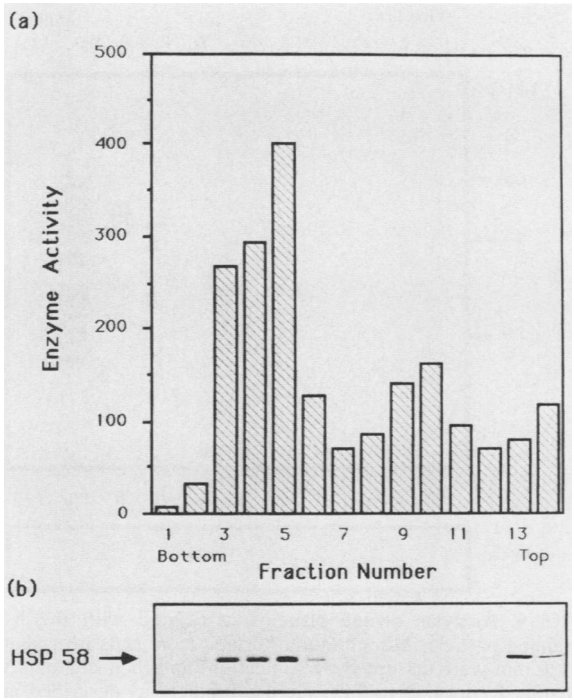

FIG. 6. Copurification of hsp58 with a mitochondria-specific enzyme. Cells were collected and mechanically disrupted, and the contents of the 2,000 × *g* supernatant of the cell lysate were sedimented into a linear 0.75 to 1.75 M isopycnic sucrose gradient as described in Materials and Methods. Each fraction of the gradient was analyzed for succinate-ferricyanide oxidoreductase activity (a) and the presence of hsp58 (b). Total enzyme activity in 300 μl of sample, measured in arbitrary units as the rate of change in the A_{420} over time, was constant for at least the first 10 min, and the cumulative change during this interval was used to determine the rates of the reactions. The distribution of hsp58 in the gradient was determined by separating proteins from each fraction of the gradient by SDS-PAGE, transferring the proteins to a nitrocellulose membrane, and probing with anti-hsp58 as described in the legend to Fig. 3. Some degree of mitochondrial damage, probably occurring during cell disruption, is indicated by the presence of succinate-ferricyanide oxidoreductase activity, which is not associated with the intact mitochondria (see fractions 9 and 10). This may also explain the hsp58 seen at the top of the gradient. Differences in the submitochondrial locations of these two components (the enzyme complex responsible for the succinate-ferricyanide oxidoreductase activity is associated with the inner membrane [54], while hsp58 appears to be located in the matrix [unpublished data]) may explain the differences in the sedimentation properties of the released succinate-ferricyanide oxidoreductase activity and hsp58.

mitochondrial protein band relative to the other mitochondrial proteins (lanes 2 and 3, Fig. 8). Thus, the data indicate that most, if not all, of the newly synthesized hsp58 induced by the heat shock accumulated in or on mitochondria.

To more carefully examine the nature of the association of hsp58 with mitochondria, we determined its resistance to proteolysis when intact or disrupted mitochondria were incubated with trypsin. The hsp58 associated with intact mitochondria was protected from proteolysis; however, when the mitochondrial membranes were disrupted with SDS, hsp58 was completely digested by the protease (Fig. 8). This indicates that hsp58 is not associated with the external surface of the mitochondria. When purified mitochondria were disrupted by sonication, most of the hsp58 fractionated with the soluble components and not with the membrane fraction (data not shown). Thus, hsp58 does not appear to be an integral membrane protein but instead is most likely either loosely associated with membranes or is a soluble protein of the mitochondria.

Further characterization of hsp58. The site of hsp58 synthesis was determined by assessing whether its synthesis during heat shock was sensitive to either chloramphenicol or cycloheximide. At a concentration of chloramphenicol (500 μg/ml) which completely inhibited cell division (53), heat-shocked cells synthesized hsp58. However, hsp58 synthesis, as well as other hsp synthesis, was not detected in cells treated with cycloheximide (5 μg/ml) during heat shock. These results indicate that hsp58 is most likely synthesized outside the mitochondrion on cytosolic ribosomes. Absolute proof of this will require identification and localization of the gene for hsp58.

hsp58 from heat-shocked cells sedimented as if it were part of a high-molecular-weight complex (Fig. 2). Although the two-dimensional electrophoretic properties of individual hsp58 from stressed and nonstressed cells were indistinguishable (data not shown), we asked whether this high-molecular-weight complex form of hsp58 was also found in the mitochondria of non-heat-shocked cells. To check this, the sedimentation of nondenatured hsp58 from heat-shocked cells was compared with that of hsp58 isolated from non-heat-shocked cells. Purified mitochondria were disrupted by sonication, and the released contents were centrifuged through a 15 to 30% sucrose gradient. Western blot analyses of the proteins in each fraction of the gradients revealed that hsp58 from the mitochondria of non-heat-shocked cells sedimented identically to that from heat-shocked cells. Similar results were obtained with extracts of mitochondria from

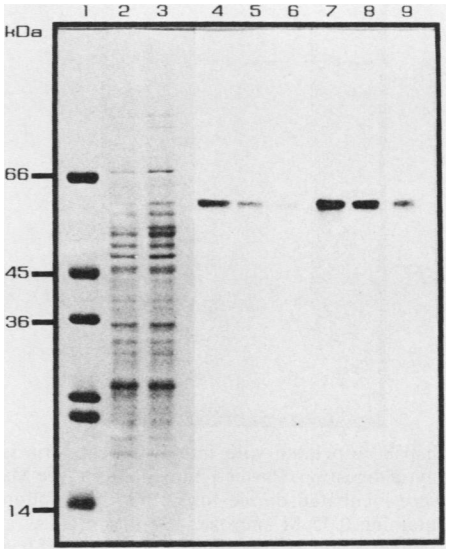

FIG. 7. Amount of hsp58 in mitochondria increases during heat shock. Mitochondrial proteins prepared from the purified mitochondria of non-heat-shocked cells (lanes 2, 4, 5, and 6) or from cells heat shocked at 41°C for 180 min (lanes 3, 7, 8, and 9) were separated by SDS-PAGE and either stained with Coomassie brilliant blue R (lanes 2 and 3) or transferred to nitrocellulose membranes and probed with the anti-hsp58 antiserum (lanes 4 through 9). Amounts of protein loaded per lane were 30 μg (lanes 2, 3, 4, and 7), 15 μg (lanes 5 and 8), and 7.5 μg (lanes 6 and 9). Quantitation of hsp58 in these samples was accomplished as described in Materials and Methods. Lane 1 contained protein size standards.

non-heat-shocked and heat-shocked cells that had been disrupted with nonionic detergents (data not shown). Treating the contents of disrupted mitochondria with RNase or β-mercaptoethanol prior to centrifugation had no effect on the sedimentation properties of the hsp58 complex (data not shown). When the proteins in each fraction of a sucrose gradient used to fractionate disrupted mitochondria were separated by gel electrophoresis and detected by silver staining, the most abundant protein in the fractions containing the hsp58 complex was a 58-kDa species (Fig. 9). Little other protein appeared to cosediment specifically and stoichiometrically with hsp58. Additionally, we could not detect any RNA molecules specifically cosedimenting with the hsp58 protein when nucleic acids, extracted from each fraction of the gradient, were separated by agarose gel electrophoresis and stained with ethidium bromide (data not shown). These results indicate that hsp58 must assemble into an oligomeric structure composed almost exclusively of that protein. If other proteins or RNA assembles with hsp58 to form the high-molecular-mass complex, they must do so in significantly submolar amounts, or they must dissociate from the complex or be degraded in the solutions used in these experiments.

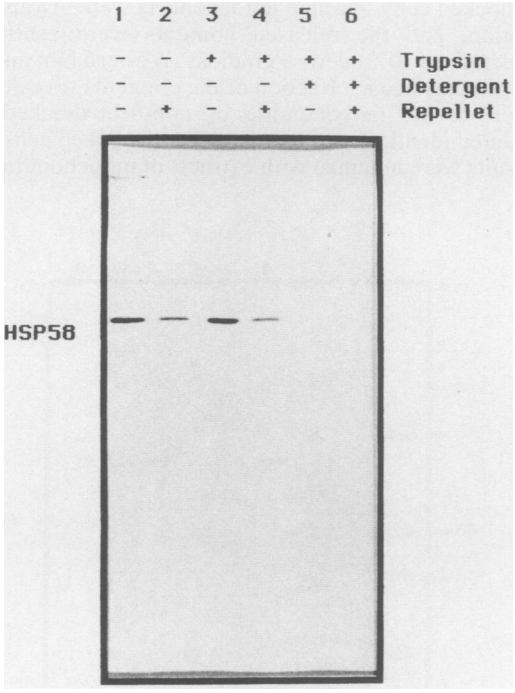

FIG. 8. hsp58 associated with intact mitochondria is protected from proteolytic digestion. Purified mitochondria (see Materials and Methods) were incubated on ice for 60 min in a buffered sucrose solution containing 0.35 M sucrose, 100 mM EDTA, and 10 mM Tris, pH 7.5 (lanes 1 and 2); the solution plus 50 μg of trypsin per ml (lanes 3 and 4); or the solution containing 50 μg of trypsin per ml and 0.1% SDS (lanes 5 and 6). At the end of the incubation period, soybean trypsin inhibitor was added to each sample at a concentration of 100 μg/ml. Half of each sample was centrifuged at 13,000 × *g* for 10 min to repellet mitochondria, which were then resuspended in the original volume of buffer (lanes 2, 4, and 6). Equal volumes of each sample were then separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the anti-hsp58 antiserum as described in the legend to Fig. 3. Because of the protocol used, the amount of mitochondrial protein run in lanes 2, 4, and 6 was about half of that run in lanes 1, 3, and 5.

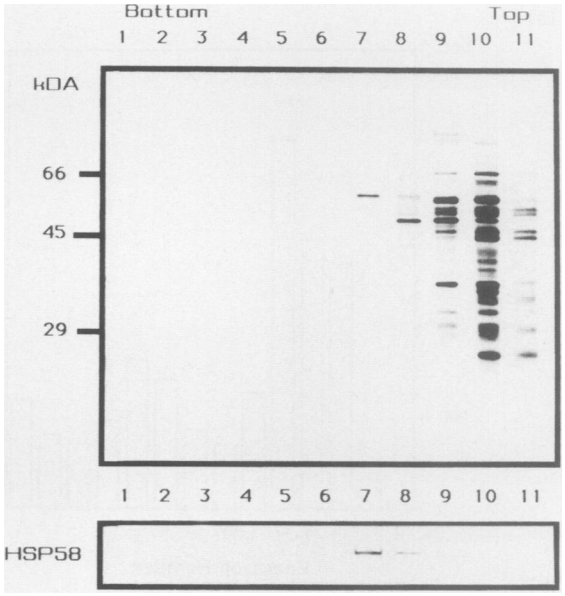

FIG. 9. Analysis of the proteins associated with the hsp58-containing particle. Mitochondria purified from cells heat shocked for 120 min were disrupted by sonication, and their contents were centrifuged into a 15 to 30% sucrose gradient as described in the legend to Fig. 2. Proteins in each gradient fraction were separated by SDS-PAGE and either silver stained (upper panel) or transferred to a nitrocellulose membrane and probed with the anti-hsp58 antiserum as described in the legend to Fig. 3 (lower panel).

DISCUSSION

We found that a normal protein component of mitochondria in non-heat-shocked *T. thermophila* displayed the same synthetic properties during heat shock as do the major hsps, namely (i) it was selectively synthesized during the early stages of heat shock, (ii) its synthesis appeared to undergo a transient increase followed by a decrease during heat shock, (iii) its overall concentration in the cell substantially increased during heat shock, and (iv) the degree of its accumulation correlated with the severity of the heat shock. The additional amount of this protein, hsp58, which accumulated during heat shock was also found to be localized within mitochondria. The association of hsps with mitochondria has been previously reported to occur in monocot and dicot plants (31, 50). The plant hsps described by Sinibaldi and Turpen (50) have sizes similar to *T. thermophila* hsp58; however, synthesis of the plant hsps was shown to occur within the mitochondria and not in the cytosol, as appears to be the case for hsp58 of *T. thermophila*.

In some organisms, cognate proteins that are structurally similar to particular hsps but whose synthesis is differentially regulated have been shown to be synthesized and accumulated in non-heat shock situations (8, 36, 41, 43, 60). If the 58-kDa protein detected in non-heat-shocked *T. thermophila* is a cognate of hsp58, then the two proteins must have no detectable size or charge difference, localize to the same subcellular compartment, and assemble into indistinguishable high-molecular-weight complexes. We believe that a more likely explanation is that hsp58 is synthesized and accumulated as a normal mitochondrial protein during nonstress conditions. In this regard, hsp58 differs from hsp73 and hsp80 in that these two major heat shock proteins are apparently in very low concentration in non-heat-shocked *T.*

thermophila cells. This statement is based on examinations of stained two-dimensional gels (17; unpublished data) and on the extremely low abundance of the mRNAs for hsp73 and hsp80 in non-heat-shocked cells (19, 20). However, since quantitative analyses of all the other hsps of *Tetrahymena* spp. have not been done, we do not know whether the normal presence of hsp58 in non-heat-shocked cells is a feature unique to this particular hsp. The fact that hsp58 exists in non-heat-shocked cells is not necessarily surprising, because studies done with other organisms have shown that several hsps are present in those organisms under nonstress conditions (9, 17, 49, 60).

The cellular abundance of hsp58 can be estimated by comparing the staining intensity of the hsp58 band with that of the 54-kDa ribosomal protein band seen in lanes 12 and 5-6, respectively, of Fig. 2b. The 54-kDa r-protein band is composed of two distinct r-proteins (21), whereas the 58-kDa band in lane 12 is a single protein species. Assuming equal recoveries of both ribosomes and hsp58 in the 100,000 × *g* pellet (as the hsp58 complex is smaller than ribosomes, hsp58 concentration is probably underestimated by this assumption) and assuming equal stain-binding properties of the proteins involved, we calculate from gel scans that there is approximately four times as much of the 54-kDa r-protein (the sum of both species) as there is of the hsp58 protein in 41°C heat-shocked cells. Since the 54-kDa r-protein species together make up approximately 1.2% of the protein in the cell (18, 37), hsp58 is estimated to represent approximately 0.3% of the total protein in heat-shocked cells and approximately 0.12% of the protein in non-heat-shocked cells. Thus, the two- to threefold increase in the concentration of hsp58 during the heat shock represents a considerable increase in the amount of this protein in the cell.

hsp58 released from disrupted mitochondria sediments in sucrose gradients as a component of a 20 to 25S complex. Since no other protein or RNA molecules were found to cosediment specifically with hsp58, this particle would appear to be composed virtually exclusively of the hsp58 protein. If this is the case, then the complex must be composed of between 12 and 16 hsp58 molecules (assuming a mass of 750,000 to 1,000,000 daltons for the particle). Electron microscopic examinations of negatively stained hsp58 complexes isolated by a variety of means from heat-shocked and non-heat-shocked cells revealed regularly arranged structures approximately 100 nm in diameter (McMullin and Hallberg, Mol. Cell. Bio., in press). The processes by which this protein is imported into mitochondria and assembled into these complexes appear to function even when the cells are subjected to hyperthermal stress.

One proposed role for hsps is to stabilize existing cellular structures against the denaturing effects of heat shock (44). Heat shock of *Tetrahymena* spp. results in a change in mitochondrial morphology (30) as well as a decrease in the ATP concentration in the cell (14, 46), indicating that mitochondrial structures and functions are directly affected by the stress. The role that hsp58 plays in the normal (i.e., nonstressed) functioning of the mitochondria is not known; however, as the major hsps, hsp73 and hsp80, do not accumulate in mitochondria during stress, one possibility is that hsp58 plays a role in mitochondria during heat shock comparable to those played by hsp73 and hsp80 in the nucleus and cytosol.

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