

# Mitochondrial Heat Shock Protein 70, a Molecular Chaperone for Proteins Encoded by Mitochondrial DNA

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**Abstract.** Mitochondrial heat shock protein 70 (mt-Hsp70) has been shown to play an important role in facilitating import into, as well as folding and assembly of nuclear-encoded proteins in the mitochondrial matrix. Here, we describe a role for mt-Hsp70 in chaperoning proteins encoded by mitochondrial DNA and synthesized within mitochondria. The availability of mt-Hsp70 function influences the pattern of proteins synthesized in mitochondria of yeast both in vivo and in vitro. In particular, we show that mt-Hsp70 acts in maintaining the var1 protein, the only mitochondrially

encoded subunit of mitochondrial ribosomes, in an assembly competent state, especially under heat stress conditions. Furthermore, mt-Hsp70 helps to facilitate assembly of mitochondrially encoded subunits of the ATP synthase complex. By interacting with the ATPase 9 oligomer, mt-Hsp70 promotes assembly of ATPase 6, and thereby protects the latter protein from proteolytic degradation. Thus mt-Hsp70 by acting as a chaperone for proteins encoded by the mitochondrial DNA, has a critical role in the assembly of supra-molecular complexes.

**T**HE biogenesis of mitochondria involves the coordinate action of both the nuclear and mitochondrial genomes. Several of the mitochondrial oligomeric enzyme complexes consist of proteins encoded by both genetic systems. Hence, the assembly into functional complexes involves the coming together of cytosolically synthesized subunits that have been imported into the mitochondria, with proteins that have been synthesized in the mitochondrial matrix (for reviews see Grivell, 1989; Tzagoloff and Dieckmann, 1990; Poyton et al., 1992). In the yeast *Saccharomyces cerevisiae*, the vast majority of mitochondrial proteins are nuclear encoded, while only eight proteins are encoded by the mitochondrial genome (Borst and Grivell, 1978; Tzagoloff and Meyers, 1986). These proteins are cytochrome *b* of the *bc*<sub>1</sub> complex; cytochrome oxidase subunits I, II, and III; subunits 6, 8, and 9 of the F<sub>0</sub>-ATP synthase (ATPase6, ATPase8, and ATPase9, respectively)<sup>1</sup> (Hadikusumo et al., 1988); and the var1 protein, a component of the ribosomal small subunit (Groot et al., 1979; Terpstra and Butow, 1979; Terpstra et al., 1979). All of these proteins are subunits of larger oligomers, and with the exception of the var1 protein, they are integral membrane proteins.

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1. *Abbreviations used in this paper:* ATPase6, ATPase8, and ATPase9, subunits 6, 8, and 9 of the F<sub>0</sub> ATP synthase, respectively; mt-Hsp70, mitochondrial heat shock protein 70; TX-100, Triton X-100.

Heat shock proteins of the Hsp70 family play essential roles as molecular chaperones in mediating intracellular protein translocation and subsequent folding and assembly (for review see Lindquist and Craig, 1988; Gething and Sambrook, 1992; Ellis, 1993; Hartl et al., 1994). Mitochondrial heat shock protein 70 (mt-Hsp70), encoded by the SSC1 gene in *S. cerevisiae*, is an essential gene product, and deletion of this gene is lethal to the yeast cell (Craig et al., 1989; Kang et al., 1990). Manipulation of the activity of this protein has been achieved through either temperature-sensitive mutants or by lowering matrix ATP levels so that they are limiting for the ATP-dependent activity of mt-Hsp70. These two approaches have been instrumental in defining two essential functions of this chaperone. Mt-Hsp70 plays a vital role in facilitating the translocation of nuclear-encoded preproteins across the mitochondrial membrane system into the matrix (Kang et al., 1990; Ostermann et al., 1990; Cyr et al., 1993; Gambill et al., 1993; Glick et al., 1993; Stuart et al., 1994a, 1994b). This function of mt-Hsp70 appears to be tightly coupled to that of the import machinery located in the mitochondrial inner membrane (Schneider et al., 1994). Furthermore, mt-Hsp70 is involved in the folding/refolding of some precursor proteins after their membrane translocation, a process that also requires the recently described mitochondrial DnaJ homologue, Mdjlp (Rowley et al., 1994).

In this report, we present evidence for a new role of mt-Hsp70, namely as a chaperone for newly synthesized proteins encoded by the mitochondrial genome. Furthermore,

we propose that the activity of mt-Hsp70 is required to prevent misfolding and, hence, aggregation of at least some of the mitochondrially encoded proteins, and by doing so, ensuring their efficient assembly, particularly under stress conditions.

## Materials and Methods

### Isolation of Mitochondria

*Saccharomyces cerevisiae* wild-type (PK82), *sscl-2* (PK81), and *sscl-3* (PK83) (Gambill et al., 1993) were grown on lactate medium (Daum et al., 1982) at 24°C and harvested at an OD<sub>578</sub> of ~1. Mitochondria were isolated as previously described (Daum et al., 1982), except that zymolyase treatment was performed at 24°C and that the purified mitochondria were finally resuspended in SEM buffer (250 mM sucrose, 1 mM EDTA, and 10 mM MOPS/KOH, pH 7.2) at a protein concentration of 10 mg/ml.

### Labeling of Mitochondrial Translation Products

In vivo labeling of mitochondrial translation products was performed after inhibition of the cytosolic protein synthesis with cycloheximide as described by Douglas and Butow (1976). Lactate medium (20 ml) was inoculated from a fresh agar plate and grown overnight at 25°C. Cells were harvested by centrifugation for 5 min at 4,000 g in a rotor (JA20; Beckman Instruments, Inc., Fullerton, CA) resuspended in yeast nitrogen base-labeling medium at an OD<sub>578</sub> of 3, shaken for 2 h, harvested again, and resuspended in yeast nitrogen base at an OD<sub>578</sub> of 5. An aliquot (250  $\mu$ l) was further incubated for 15 min at either 25°C or 37°C, as indicated. Cycloheximide (150  $\mu$ g/ml final concentration) was then added, incubation continued for 1 min, and then 8  $\mu$ l of a mixture of all amino acids except methionine (2 mg/ml each) and 20  $\mu$ Ci of [<sup>35</sup>S]methionine (1,000 Ci/mmol) were added. The cells were further incubated with shaking for 10 min, then 10  $\mu$ l stop-mixture (0.1 M methionine, 13 mg/ml chloramphenicol) was added. Total cell proteins were extracted by TCA precipitation and were solubilized by shaking at 4°C for 30 min in LiDS sample buffer (2% lithium dodecylsulfate, 10% glycerol, 2.5%  $\beta$ -mercaptoethanol, 0.02% bromophenolblue, and 60 mM Tris/HCl, pH 6.8). Proteins were separated by SDS-PAGE and were visualized by fluorography (Laemmli, 1970).

In vitro labeling of mitochondrial translation products was performed as described previously (McKee and Poyton, 1984; Herrmann et al., 1994). Unless otherwise indicated, samples (30  $\mu$ l vol) consisted of isolated mitochondria (40  $\mu$ g protein) incubated in translation buffer (0.6 M sorbitol, 150 mM KCl, 15 mM KH<sub>2</sub>PO<sub>4</sub>, 13 mM MgSO<sub>4</sub>, 20 mM Tris/HCl, 0.15 mg/ml of all amino acids except methionine, 4 mM ATP, 0.5 mM GTP, 5 mM  $\alpha$ -ketoglutarate, 5 mM phosphoenolpyruvate, and 3 mg/ml fatty acid-free BSA, pH 7.4) containing 0.6 U pyruvate kinase and 10  $\mu$ Ci [<sup>35</sup>S]methionine. Samples were incubated for 20 min at 30°C, after which labeling was stopped by adding cold methionine to a final concentration of 25 mM and incubating further for 5 min. Mitochondria were reisolated, washed once in 500  $\mu$ l 0.6 mM sorbitol, 1 mM EDTA, and 5 mM methionine, pH 7.2 (washing buffer), and lysed in 25  $\mu$ l LiDS sample buffer.

### Coimmunoprecipitation Experiments

After in vitro labeling in 40  $\mu$ g isolated mitochondria for 20 min, apyrase (40 U/ml) and oligomycin (20  $\mu$ M) were added. Samples were incubated further at 30°C for 8 min, then unlabeled methionine was added at a final concentration of 0.5 M, and incubation was continued for 2 min. The mitochondria were reisolated, washed in washing buffer, and lysed for 10 min at 4°C in 200  $\mu$ l of 0.1% Triton X-100 (TX-100) lysis buffer (0.1% TX-100 [wt/vol], 150 mM NaCl, 10 mM Tris/HCl, 5 mM EDTA, 1 mM PMSF, and 20 U/ml apyrase, pH 7.4). After a clarifying spin for 10 min at 20,000 g in a Beckman JA18.1 rotor, the supernatant was added to 2 mg protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ), to which the immunoglobulin fraction from 30  $\mu$ l preimmune or specific antiserum raised against Ssc1p or the  $\alpha$  subunit of the F<sub>1</sub>-ATP synthase had been bound, as indicated. The suspension was gently shaken for 1 h at 4°C, and the Sepharose beads were collected by centrifugation in an Eppendorf centrifuge, washed twice with lysis buffer, once with 10 mM Tris/HCl, pH 7.4, and were finally resuspended in LiDS sample buffer. After shaking for 30 min at 4°C, the beads were pelleted by centrifugation, and the eluted proteins in the supernatant were subjected to SDS-PAGE and fluorography.

## Fractionation of Mitochondria

**Determination of Aggregated Translation Products.** After labeling of proteins in 100  $\mu$ g isolated mitochondria, the mitochondria were reisolated, washed in washing buffer, and lysed for 10 min at 4°C in 250  $\mu$ l 0.1% TX-100-lysis buffer. Aggregates were pelleted by centrifugation at 30,000 g in a Beckman JA18.1 rotor for 15 min and resuspended in sample buffer. Soluble proteins in the supernatant were precipitated by addition of 50  $\mu$ l of 72% TCA (wt/vol), collected by centrifugation, washed with cold acetone, and dissolved in sample buffer. The translation products were analyzed by SDS-PAGE and were visualized by fluorography.

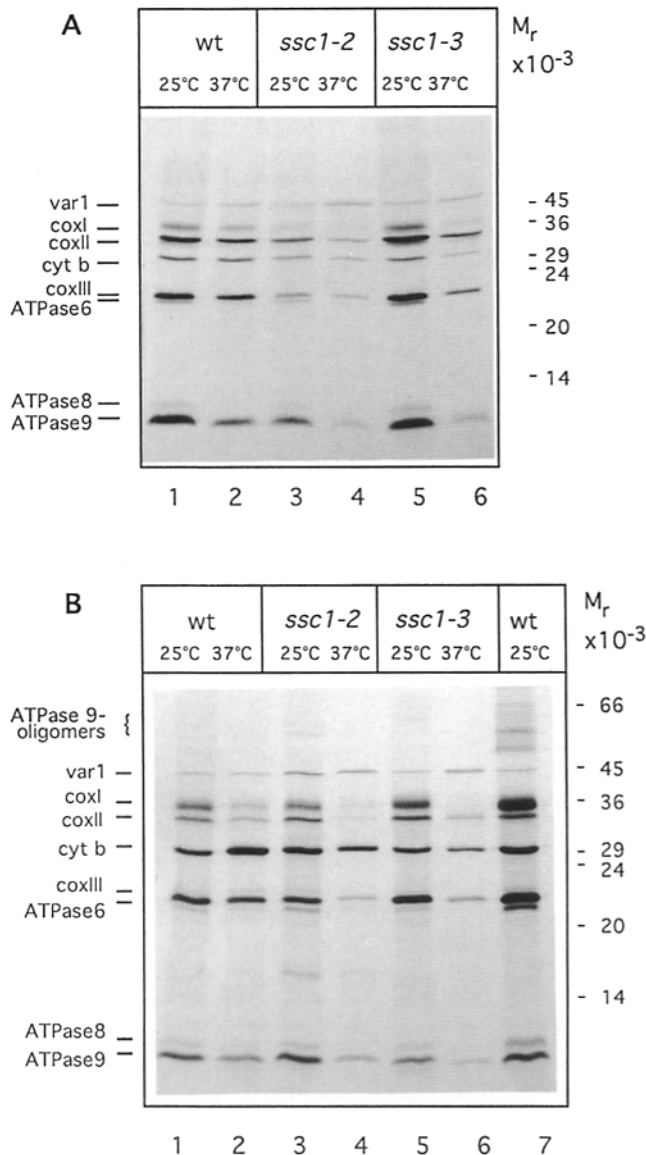
**Isolation of Ribosomes.** Freshly isolated mitochondria were solubilized in AMT<sup>500</sup> buffer (2% Triton X-100 [wt/vol] in 500 mM NH<sub>4</sub>Cl, 10 mM MgSO<sub>4</sub>, 6 mM  $\beta$ -mercaptoethanol, and 10 mM Tris/HCl, pH 7.4) at a protein concentration of 5 mg/ml for 10 min at 0°C. After a clarifying spin at 30,000 g in Beckman JA18.1 rotor for 15 min, the ribosomes were sedimented by centrifugation in a Beckman TL100 ultracentrifuge in a TL100.3 rotor at 540,000 g for 1 h. The resulting ribosomal pellet was resuspended in 200  $\mu$ l AMT<sup>500</sup> and was loaded on a continuous 12-ml 10–34% (wt/vol) sucrose gradient in AMT<sup>500</sup> and centrifuged for 15 h at 100,000 g at 4°C in a Beckman SW41 rotor. 750- $\mu$ l fractions were collected, TCA precipitated, and dissolved in LiDS sample buffer. The presence of ribosomal particles was analyzed by pumping a parallel gradient of unlabeled material through a continuous flow cell of a Kontron spectrophotometer and recording the absorbance at 260 nm.

## Results

### Mitochondrial Translation Continues in Mutants with Defective mt-Hsp70 Function, but Results in Altered Pattern of Proteins Synthesized

To investigate the possible involvement of mt-Hsp70 in the translation of mitochondrial-encoded proteins, we used two temperature-sensitive yeast strains containing mutations in the *SSC1* gene, the *sscl-2* and *sscl-3* mutants (Kang et al., 1990; Gambill et al., 1993). Mitochondrial protein synthesis in these mutants was analyzed initially in vivo after the inactivation of mt-Hsp70 by exposure to nonpermissive temperature (Fig. 1 A). Yeast cells grown at 25°C were either maintained at this temperature or shifted to the nonpermissive temperature of 37°C. Cycloheximide was then added to block cytosolic protein synthesis, and [<sup>35</sup>S]methionine was added to label proteins synthesized on mitochondrial ribosomes. At 25°C, practically the same pattern of labeling was observed in all cell types. At 37°C, the pattern of labeled mitochondrial proteins in all cell types was slightly altered in comparison to translation at 25°C. These differences were more pronounced in the *sscl* mutants, where a reduction in particular of subunits I–III of cytochrome oxidase was observed in comparison to wild-type cells.

Similar results were obtained in translation studies performed with isolated mitochondria. In this case, the isolated mitochondria were either kept at 25°C or exposed to 37°C before their energization for labeling (Fig. 1 B). No major differences in the translation products were observed if translation was performed at 25°C between mitochondria from the various strains; however, there was a strong reduction in the formation of most proteins at 37°C in the *sscl* mutants. Some translation products, such as cytochrome oxidase subunit I, ATPase<sub>6</sub>, and ATPase<sub>8</sub>, were almost absent at elevated temperatures, especially in the two mt-Hsp70 mutants. In contrast, the labeling of the var1 protein, a component of the ribosomal small subunit, was not affected and indeed appears to be increased at 37°C, especially in the mitochondria from both mutants. In addition, we observed two high molecular mass oligomers (indicated ATPase



**Figure 1.** Patterns of mitochondrial protein synthesis in vivo and in vitro in wild-type and the *ssc1* mutants. (A) Yeast cells of wild-type (*wt*) (lanes 1 and 2), *ssc1-2* (lanes 3 and 4), and *ssc1-3* (lanes 5 and 6) were grown on lactate medium overnight at 25°C. Cells were then transferred to 37°C for 15 min (lanes 2, 4, and 6) or kept at 25°C (lanes 1, 3, and 5). Cycloheximide was added, followed by amino acids and [<sup>35</sup>S]methionine. After further incubation for 10 min, labeling was stopped, and total cellular proteins were extracted and pelleted by TCA precipitation, resolved by SDS-PAGE, and visualized by fluorography, as described in Materials and Methods. (B) Isolated mitochondria (40 μg protein) from either *wt* (lanes 1, 2, and 7), *ssc1-2* (lanes 3 and 4), or *ssc1-3* cells (lanes 5 and 6) were resuspended in translation buffer and preincubated for 10 min at 25°C (lanes 1, 3, 5, and 7) or at 37°C (lanes 2, 4, and 6). Translation was monitored as described in Materials and Methods after the addition of [<sup>35</sup>S]methionine for 30 min at 25°C or 37°C, as indicated. Labeling was stopped by addition of excess unlabeled methionine, and samples were incubated for an additional 5 min. The mitochondria were isolated, washed, and either TCA precipitated before solubilization in LiDS sample buffer (lanes 1–6) or resuspended directly in LiDS sample buffer (lanes 7). Proteins were separated on SDS-PAGE and visualized by fluorography. *coxI*, *coxII*, and *coxIII*, subunits I, II, and III of the cytochrome oxidase complex, respectively; *cyt b*, cytochrome *b*. The 48- and 54-kD oligomers of the ATPase9 are referred to as ATPase9 oligomers. The positions of molecular mass markers (*kD*) are indicated.

oligomers, see below) of 48 and 54 kD (Fig. 1, lane 7). Such oligomers were dissociated by TCA precipitation since they were only observed in the absence of such treatment (Fig. 1 B, lane 1 vs lane 7). The formation of the larger of these oligomers appeared to be affected in the mutant mitochondria (see below).

These results together would suggest that mt-Hsp70 function is not absolutely required for the translation process per se, but that it has distinct effects on the quantities of proteins synthesized and possibly their stability. It should be pointed out, however, that the *ssc1* mutants defective in mitochondrial protein import may not be affected in all functions of mt-Hsp70 and, therefore, a residual activity in supporting mitochondrial translation cannot be excluded.

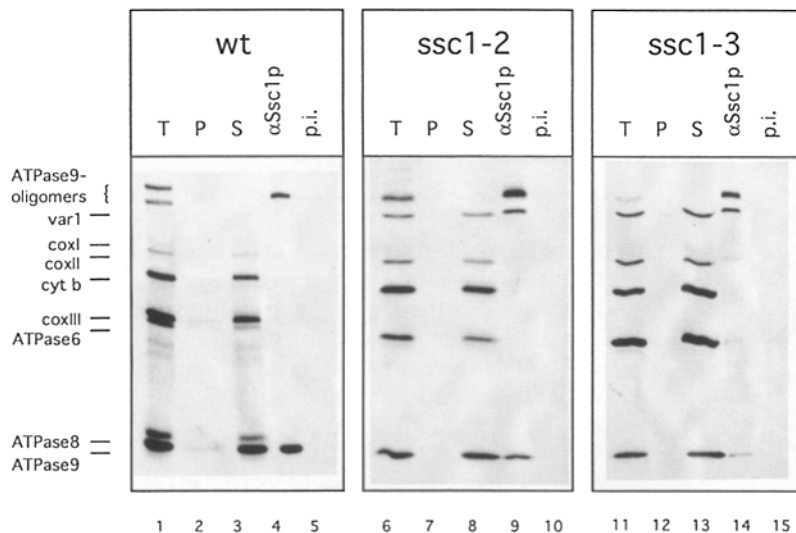
### Mt-Hsp70 Interacts with Newly Synthesized Mitochondrially Encoded Proteins

We addressed whether the newly synthesized proteins interacted with mt-Hsp70 before their assembly either during or after their synthesis. After translation in organello, mitochondria were lysed with detergent under conditions that ensured solubilization of membrane-assembled proteins. Physical interaction of the solubilized proteins with mt-Hsp70 was tested by coimmunoprecipitation studies (Fig. 2). In both wild-type mitochondria and in the *ssc1* mutants, mt-Hsp70 was found in association selectively with the *var1* protein and with ATPase9 (Fig. 2, lane 4). In the case of the latter protein, preferentially the oligomeric form constituting a 48-kD complex of the ATPase9 was observed in contact with mt-Hsp70. It seems likely that the monomeric form of ATPase9 that was detected resulted from dissociation of the 48-kD complex. The 48-kD complex was observed to partly dissociate upon electrophoresis, and the amount of ATPase9 coimmunoprecipitated with mt-Hsp70 correlated with the amount of 48-kD complex present rather than with the amount of monomeric form.

The efficiency of coimmunoprecipitation of both *var1* and ATPase9 was significantly higher in the *ssc1-2* mutant in comparison to the wild type. This observation is consistent with the proposal that the *ssc1-2* mutant mt-Hsp70 retains the ability of binding to substrates, whereas the release is suggested to be impaired (Fig. 2, lane 9). Very efficient coimmunoprecipitation with mt-Hsp70 was also observed in the *ssc1-3* mutant mitochondria (Fig. 2, lane 14). This finding indicates that this mutant mt-Hsp70 has retained the capacity to bind mitochondrially encoded substrates, although in vitro import of nuclear-encoded preproteins, a process requiring cyclical binding of mt-Hsp70 to incoming polypeptide chains, was found to be completely blocked in this mutant (Gambill et al., 1993).

Thus, mt-Hsp70 appears to physically interact with at least two of the newly synthesized proteins, namely the *var1* and the subunit 9 of the ATPase.

dase complex, respectively; *cyt b*, cytochrome *b*. The 48- and 54-kD oligomers of the ATPase9 are referred to as ATPase9 oligomers. The positions of molecular mass markers (*kD*) are indicated.



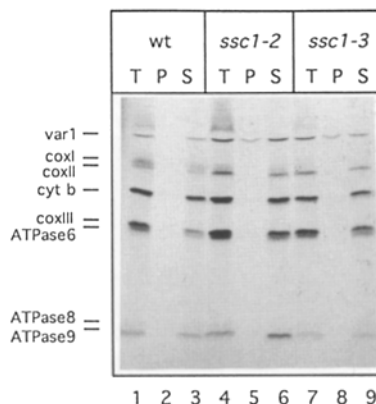
Ssc1p antiserum (lanes 4, 9, and 14) or preimmune serum (lanes 5, 10, and 15), as described in Materials and Methods. All samples were analyzed by SDS-PAGE and fluorography. The amount of label depicted in the total pellet and supernatant samples corresponds to 10% of that used for the coimmunoprecipitations. *T*, total; *S*, supernatant; *P*, pellet;  $\alpha$ Ssc1p, antiserum raised against Ssc1p; *p.i.*, pre-immune serum.

### Mt-Hsp70 has a Role in Maintaining var1 in a State Competent for Assembly into Ribosomes

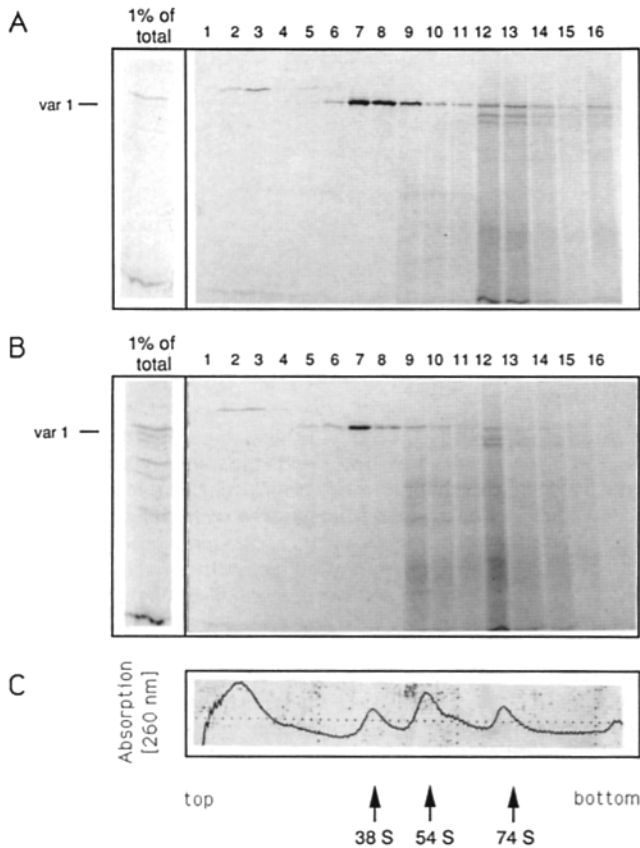
We then asked whether the function of mt-Hsp70 was to interact with the var1 protein before its assembly into ribosomes. To address this question, after the labeling of translation products, mitochondria were solubilized with Triton X-100, and the formation of aggregates was analyzed. Of the newly synthesized proteins, var1 protein was the only one in which a significant proportion was found aggregated in both the *ssc1-2* and *ssc1-3* mutant mitochondria, but not in wild-type mitochondria (Fig. 3). The presence of var1 in this aggregate fraction did not result from pelleted ribosomes (not shown). The var1 remaining in the soluble fraction consisted of two distinct populations, namely var1 assembled into ribosomes, which was not pelleted under the centrifugation conditions used and an unassembled fraction that remained soluble (see below).

For analyzing in more detail the assembly of var1, yeast cells that had been exposed to chloramphenicol before isolating mitochondria were used. This treatment was aimed at increasing the pools of preribosomal complexes, whose protein components are entirely nuclear encoded (Maheshwari and Marzuki, 1985). Newly synthesized var1 could assemble into functional ribosomes in wild-type mitochondria, as verified by sucrose gradient centrifugation of a mitochondrial ribosomal pellet fraction (Fig. 4 A). The radiolabeled var1 mainly comigrated with the small ribosomal subunits, and only to a minor extent with complete ribosomes that were active in translation, as judged by the presence of radiolabeled nascent chains. Assembly of var1 occurred also in the *ssc1-3* mitochondria, with an efficiency slightly less than that observed in the wild-type mitochondria (Fig. 4, A and B). These results indicate that after synthesis, var1 is competent for assembly, and they suggest that this process apparently does not depend directly on the activity of mt-Hsp70. Assembly of var1 was observed also in *ssc1-2* mitochondria (results not shown). The level of assembled var1 achieved under

these in vitro conditions presumably reflects the levels of preribosomal complexes existing. Indeed, if the chloramphenicol pretreatment was omitted, the level of var1 assembly into the ribosomes was significantly reduced (results not shown). Taken together, these results indicate that the newly synthesized var1 can assemble, to a certain extent, into func-



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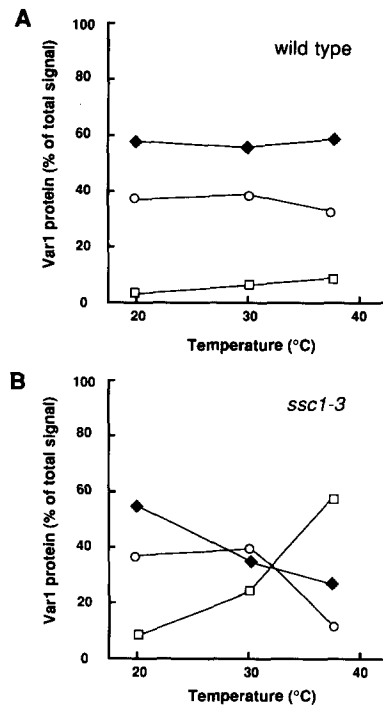


**Figure 4.** Assembly of in vitro translated var1 into ribosomal precomplexes. Wild-type and *ssc1-3* cells were grown in lactate medium and were treated with chloramphenicol (4 mg/ml) for 2 h before harvesting and subsequent mitochondria isolation. Isolated mitochondria (8 mg) were incubated at 37°C for 10 min in 1.6 ml translation buffer before transferring them to 30°C and adding of 150  $\mu$ Ci [ $^{35}$ S]methionine for 1 h. After translation, the mitochondria were reisolated, washed, and extracted in AMT<sup>500</sup> buffer at a protein concentration of 5 mg/ml. Ribosomes were then resolved on sucrose density gradients, as described in Materials and Methods. Fractions were collected, TCA precipitated, dissolved in sample buffer, electrophoresed, and fluorographed. The panels to the left show 1% of total translation signal. (A) Wild-type mitochondria. (B) *ssc1-3* mitochondria. (C) A sucrose gradient on which ribosomes from wild-type mitochondria were resolved was analyzed by recording the absorption at 260 nm during flow through a quartz cell. The positions of the ribosomal subunits and monomer are indicated.

tional ribosomes under the in vitro conditions used here. However, a significant proportion of the var1 in wild-type mitochondria remains unassembled, and it is maintained as a soluble species. In the apparent absence of mt-Hsp70 function, a large part of the newly synthesized var1 aggregates. Thus, the function of mt-Hsp70 appears to be required to maintain unassembled var1 in a soluble state.

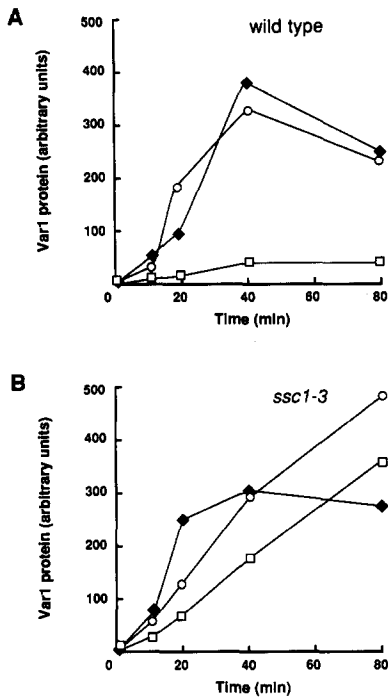
#### ***Mt-Hsp70 Protects var1 against Aggregation at Elevated Temperatures***

Mitochondria isolated either from wild-type or from *ssc1-3* mutant cells were exposed to the nonpermissive temperature, then labeling of mitochondrial translation products was performed in the presence of [ $^{35}$ S]methionine at tempera-



**Figure 5.** Aggregation of var1 in wild-type and *ssc1-3* mutant mitochondria at higher temperatures. Isolated mitochondria (2.7 mg protein) from chloramphenicol-treated wild-type (A) or *ssc1-3* cells (B) were resuspended in 550  $\mu$ l translation buffer and incubated at 37°C for 10 min. After the addition of [ $^{35}$ S]methionine, the samples were divided into three parts, and translation was performed at either 20°C, 30°C, or 37°C for 40 min. Samples were divided, and mitochondria were reisolated from both halves and were washed. In one case, the mitochondria were directly lysed in LiDS sample buffer for the analysis of total signal. The other half was used to analyze the distribution of var1 between submitochondrial fractions. These samples were lysed in 150  $\mu$ l AMT<sup>500</sup> buffer for 10 min on ice. Aggregated material in this detergent extract was pelleted by centrifugation for 10 min at 30,000 g. The resulting supernatant was then recentrifuged for 1 h at 436,000 g to recover the ribosomes. The proteins were electrophoresed and visualized by fluorography. Resulting films were quantified by laser densitometry, and the levels of var1 present in the soluble fraction (O), in the aggregated material ( $\square$ ), or assembled into ribosomes ( $\blacklozenge$ ) are expressed as percentage of total signal.

tures of 20°C, 30°C, and 37°C. Mitochondria were lysed with detergent and subfractionated into aggregated material, ribosomal fraction, and soluble fraction. The distribution of the newly synthesized var1 between these fractions was then monitored (Fig. 5). Efficient assembly of var1 into ribosomes (~60% of total signal) was achieved in wild-type mitochondria after synthesis at all temperatures studied (Fig. 5 A). The remaining fraction of var1, which represented the unassembled form, was almost exclusively recovered as a soluble species. Only a minor proportion was recovered as aggregates after translation at all temperatures analyzed. In the *ssc1-3* mitochondria, after synthesis at lower temperatures (20°C), as in wild-type mitochondria, a large proportion of the var1 became efficiently assembled into ribosomes (Fig. 5 B). The remainder of the newly synthesized var1 was recovered in the soluble fraction with a small percentage (~10%) being found in an aggregated form. At elevated tem-

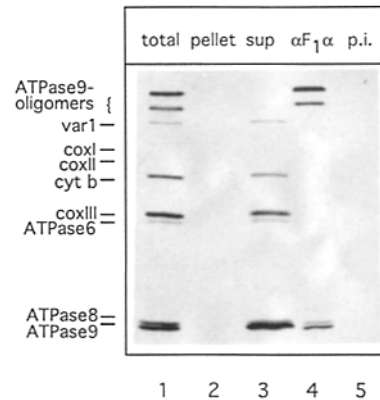


**Figure 6.** Kinetics of var1 assembly, aggregation, and degradation in wild-type and *ssc1-3* mutant mitochondria. Isolated mitochondria (3.5 mg protein) from chloramphenicol-treated wild-type (A) or *ssc1-3* (B) cells were resuspended in translation buffer and transferred to 37°C for 10 min. [<sup>35</sup>S]Methionine was then added, and the samples were divided into four parts and labeled at 30°C for 10, 20, 40, or 80 min. After translation, samples were divided, and the proportions of var1 present in the soluble fraction (○), aggregated material (□), or assembled into ribosomes (◆) were determined as described in Fig. 5 and are expressed as arbitrary units.

peratures, however, the levels of both assembled and, particularly, of soluble (unassembled) var1 decreased markedly, and a concomitant increase in aggregated var1 was observed.

Despite the fact that the *ssc1-3* mitochondria were preincubated at 37°C to inactivate the mt-Hsp70, there was a very high proportion of soluble and assembly-competent var1 observed if translation was performed at 20°C. Newly synthesized var1 may remain in a soluble form without the assistance of chaperones if maintained at low temperatures, or other mitochondrial chaperones could substitute for mt-Hsp70 at lower but not at higher temperatures. Alternatively, the mt-Hsp70 activity in the *ssc1-3* mutant may be insufficient to support protein import after exposure to nonpermissive temperature, but it could be sufficient to stabilize proteins under nonstress conditions. The competence of this mutant mt-Hsp70 to still bind to var1 (see Fig. 2, lane 14) after its exposure to 37°C supports this notion.

The kinetics of aggregation of the unassembled var1 was then studied in the wild-type mitochondria and in the *ssc1-3* mitochondria (Fig. 6). Both sets of mitochondria were preincubated at 37°C. Samples were then returned to 30°C, where mitochondrial translation was initiated and the fate of the var1 protein was monitored during the time periods indicated. In wild-type mitochondria, the newly synthesized var1 protein predominantly partitioned between the ribosomal and the soluble protein fractions, over the whole time



**Figure 7.** Both the 48 kDa and 54 kDa oligomers can form a complex with the  $\alpha$ -subunit of the  $F_1$ -ATPase. Wild-type cells were grown in lactate medium to which chloramphenicol (4 mg/ml) was added 2 h before harvesting. Mitochondria were isolated, and they were labeled with [<sup>35</sup>S]methionine, as described in Fig. 1. The samples were divided, one part was directly dissolved in LiDS sample buffer (lane 1, total), and the rest was lysed in 0.1% T X-100 lysis buffer and then centrifuged at 20,000 g for 10 min. The pellet was resuspended in LiDS sample buffer (lane 2, pellet), and the supernatant was divided in three parts. One part was directly TCA precipitated (lane 3, sup), while the others were added to protein A-Sepharose beads to which antiserum against  $F_1\alpha$  (lane 4) or preimmune serum (lane 5) had been coupled and further processed as described in Materials and Methods. The amount of label depicted in the total, pellet, and supernatant samples corresponds to 10% of that used for the coimmunoprecipitations.

course period studied (Fig. 6 A). Aggregated var1 always represented a minor fraction of the total signal (<10%). In contrast, in the *ssc1-3* mitochondria, a time-dependent aggregation of a portion of var1 took place (Fig. 6 B).

Furthermore, in wild-type mitochondria, both the soluble and assembled forms of var1 reached a maximum after 40 min, and degradation was observed at later time points. In contrast, in the *ssc1-3* mitochondria, the total var1 signal increased in nearly a linear fashion over all time points studied; apparently, proteolytic degradation did not occur, or did so to a much lesser extent, in the *ssc1-3* mitochondria. In turn, these findings suggest mt-Hsp70 is involved in facilitating degradation of var1 in mitochondria. Indeed, an increasing amount of evidence favors a general function of mt-Hsp70 in mediating degradation of imported proteins (Wagner et al., 1994). It is unclear whether all nonassembled, mitochondrially encoded proteins are degraded by the same proteolytic system. Newly synthesized ATPase6 was degraded more extensively in the mt-Hsp70 mutant mitochondria than in the wild-type situation (see below). Therefore, mt-Hsp70 activity appears not to be required in facilitating the proteolytic degradation of this membrane protein.

#### **A Role for mt-Hsp70 in Stabilizing Newly Synthesized Subunits of the ATP Synthase**

A number of high molecular weight complexes of mitochondrial gene products were observed after synthesis in isolated mitochondria (see Fig. 1 B, lane 7). These complexes were resistant to disruption by SDS; however, if the samples were TCA precipitated before SDS electrophoresis, these com-

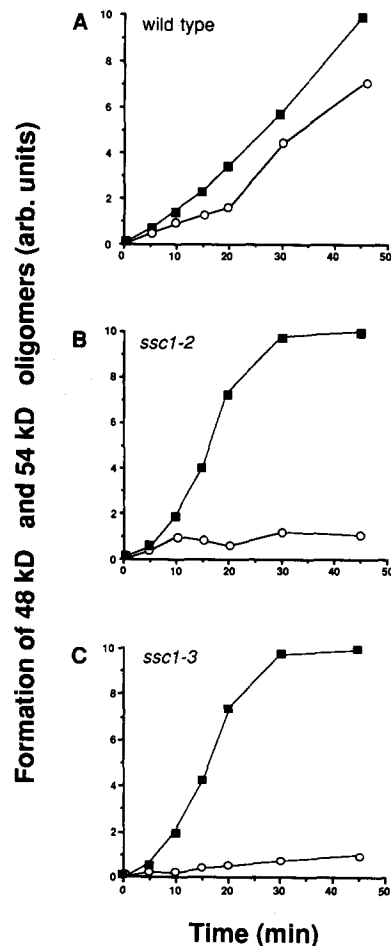
plexes were almost completely dissociated (Fig. 1 *B*, lane 1). The two major complexes of 48 and 54 kD are composed of subunits of the F<sub>0</sub> part of the ATP synthase. The 48-kD complex, which represents the ATPase9 oligomer (Tzagoloff and Akai, 1972; Tzagoloff et al., 1973), in contrast to the 54-kD complex, could be coimmunoprecipitated with antibodies against mt-Hsp70 (see Fig. 2). If this coimmunoprecipitated 48-kD oligomer was treated with TCA, and then analyzed by SDS-PAGE, monomers of ATPase9 were recovered (results not shown). Furthermore, we could demonstrate that both the 48- and 54-kD oligomers (or at least a subpopulation of them) could also exist in a complex with the  $\alpha$  subunit of the F<sub>1</sub> part of the ATPase because they could be coimmunoprecipitated with an antibody against this protein (Fig. 7, lane 4). If this coimmunoprecipitate was also treated with TCA, both monomers of ATPase6 and ATPase9 were released (results not shown), suggesting the 54-kD oligomer contained both ATPase subunits. This was confirmed in an independent approach where the excised 54-kD band treated with TCA and resubjected to electrophoresis also gave rise to both ATPase6 and ATPase9 (results not shown).

We asked whether the 48-kD oligomer represented an assembly intermediate of the ATP synthase whose interaction with mt-Hsp70 was necessary for further assembly of complex formation, namely to the 54-kD oligomer. The formation of the 54-kD oligomer in relation to the 48-kD oligomer was monitored in both wild-type mitochondria and the *sscl* mutant mitochondria, after their exposure to nonpermissive temperature (Fig. 8). Formation of the ATPase9 oligomer (the 48-kD complex) occurred in both wild-type and mutant mitochondria. In contrast, the 54-kD complex was observed at significant levels only in the wild-type mitochondria (Fig. 8 *A*).

Thus, the formation of the 48-kD complex does not require mt-Hsp70 function; however, the efficient further assembly of the ATPase6 into this complex resulting in the formation of the 54-kD one is dependent on mt-Hsp70 action. These findings are consistent with the previous observation that the 48-kD complex could be coimmunoprecipitated with mt-Hsp70, while the 54-kD could not.

In summary, assembly of the ATPase6 into a complex containing ATPase9 appears to be affected in the *sscl* mutant mitochondria. In support of this, we observed a distinct instability of the ATPase6 subunit after synthesis in the *sscl* mutant in contrast to the wild-type situation (Fig. 9). Mitochondria prepared from chloramphenicol-treated, wild-type and *sscl-3* yeast cells were exposed to 37°C before translation at 30°C. The fate of the ATPase6 was followed during both the pulse and the subsequent chase period. ATPase6 levels were normalized to those of cytochrome *b* synthesized, a protein that is observed to be very stable in both wild-type and *sscl* mutant mitochondria. In wild-type mitochondria, labeled ATPase6 accumulated during the pulse period; however, degradation (~50%) was observed in the beginning of the chase period. The remaining 50% of ATPase6 was stable and presumably reflects the assembled form of the protein. In contrast, in the *sscl-3* mitochondria, the newly synthesized ATPase6 was extremely unstable with significant degradation occurring even during the pulse period.

Taking all these results together, we conclude that mt-



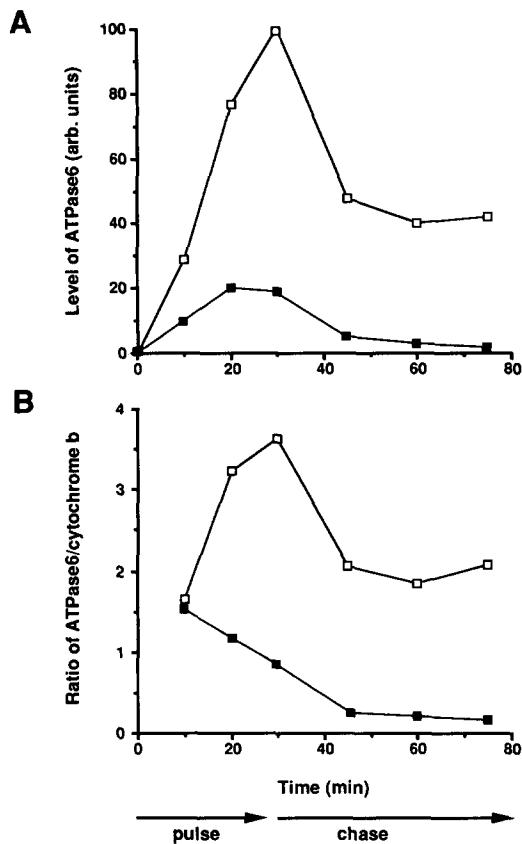
**Figure 8.** Formation of the 48- and 54-kD oligomers of ATPase9 in wild-type and *sscl* mutants. Isolated mitochondria (35  $\mu$ g) from chloramphenicol-treated wild-type, *sscl-2*, or *sscl-3* cells were resuspended in translation buffer incubated at 37°C for 10 min and then transferred to 30°C. [<sup>35</sup>S]Methionine was then added, and after incubation for times indicated, samples corresponding to 5  $\mu$ g of mitochondria were removed and mixed with excess unlabeled methionine to prevent further labeling. The mitochondria were reisolated, washed, and resuspended in LiDS sample-buffer. Samples were electrophoresed, and the resulting gel was fluorographed. The 48- (■) and 54-kD (○) bands were quantified by densitometry. (A) Wild type. (B) *sscl-2* mutant. (C) *sscl-3* mutant.

Hsp70 plays an important role in the assembly of the ATP synthase, in particular at the level of the insertion of the ATPase6 into this complex. If such an event in the assembly process is adversely affected, as was the case in the *sscl* mutants where the formation of the 54-kD oligomer is blocked, the resulting unassembled ATPase6 monomers are highly unstable and are rapidly degraded.

## Discussion

Mitochondrial protein translation is strongly influenced by the availability of functional mt-Hsp70. Although it is not easy to determine how direct the influence of mt-Hsp70 is with each protein synthesized, this molecular chaperone certainly plays a decisive role in the expression of mitochondrial genes. In a few cases, we have analyzed the role of mt-Hsp70

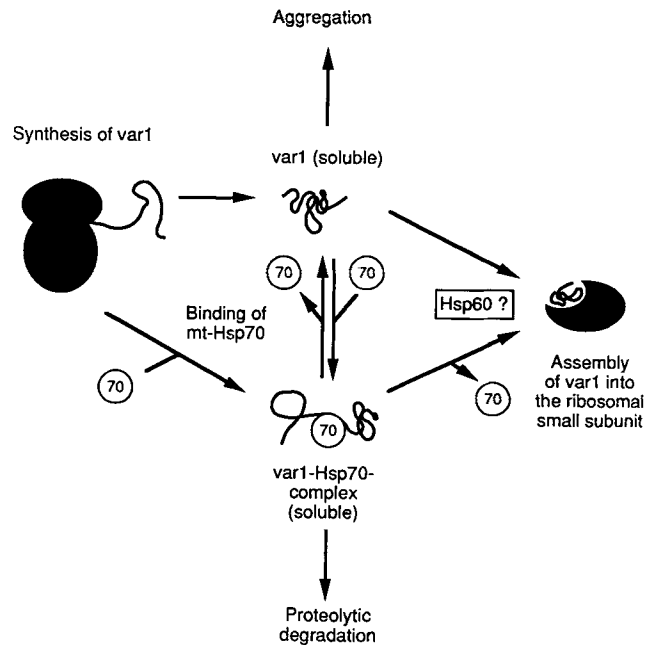




**Figure 9.** Proteolytic degradation of ATPase6 synthesized in isolated mitochondria. Wild-type and *sscl-3* cells were grown in lactate medium to which chloramphenicol (4 mg/ml) was added 2 h before harvesting. Mitochondria were isolated, treated for 10 min at 37°C, and labeled with [<sup>35</sup>S]methionine for 30 min at 30°C (termed pulse), as described in Fig. 1. Then puromycin (20 μg/ml) and unlabeled methionine were added, and the mitochondria were incubated for another 45 min. During this period (termed chase) at times indicated, aliquots were taken, the mitochondria were reisolated, and radioactively labeled products were analyzed by electrophoresis, fluorography, and densitometry of resulting bands. The relative amounts of ATPase6 were plotted (A) or were related to the cytochrome *b* signal at each given time point (B). Wild-type (□), *sscl-3* (■).

in some detail. Mt-Hsp70 can undergo a direct interaction with some mitochondrial translation products and thereby support their subsequent assembly, particularly under thermal stress conditions. How does mt-Hsp70 act on mitochondrially encoded proteins to influence their assembly competence? Most likely, it does not influence the reaction in a specific manner, but rather, by interacting with proteins of certain conformation, mt-Hsp70 affects the equilibrium of reactions. By doing so, mt-Hsp70 can prolong the lifetime of certain states of a protein, e.g., of an assembly-competent state. As a result of this chaperoning function, mt-Hsp70 does not take part in the assembly process directly, but it promotes the overall reaction.

In this particular study, we have concentrated on the var1 protein, the only hydrophilic nonmembrane protein encoded by a mitochondrial gene in *S. cerevisiae* and on two subunits of the ATP synthase, ATPase6 and ATPase9, two firmly integrated membrane proteins. The interaction of mt-Hsp70



**Figure 10.** Working model for the role of *sscl* in assembly, aggregation, and degradation of the var1 protein. See text for details.

with the var1 protein is coupled with the assembly process of this ribosomal protein, as proposed in Fig. 10. Var1 is synthesized on mitochondrial ribosomes, and it has the potential to assemble into preribosomal complexes whose protein content is entirely nuclear encoded. After synthesis, var1 exists as a soluble protein in the mitochondrial matrix, and from there, its destiny is determined by a number of factors, namely availability of preribosomal complexes, presence of functional mt-Hsp70, and the activity of the proteolytic degradation system. In wild-type mitochondria, if assembly is limiting, e.g., because of insufficient ribosomal precomplexes, the residual unassembled var1 is stabilized as a soluble species through an interaction with mt-Hsp70. Such an interaction is particularly necessary under stress conditions of elevated temperatures. In addition, this unassembled var1 appears to be susceptible to proteolytic degradation. It is not clear which mitochondrial protease is responsible for this process; however, it was not observed in the *sscl* mutants and, hence, appears to require a functional mt-Hsp70 to facilitate it. The recently identified PIM1 protease (Kutejová et al., 1993; van Dyck et al., 1994) may be involved in this degradation process. Proteolytic breakdown by this protease was recently found to depend on mt-Hsp70 activity (Wagner et al., 1994).

In the apparent absence of the chaperone activity of mt-Hsp70 in the *sscl* mutants, the labile nature of the newly synthesized var1 was more pronounced. At higher temperatures, the var1 protein displayed a distinct tendency to form aggregates. Formation of such aggregates had profound effects on both the solubility and assembly competence of the var1 protein. We propose that mt-Hsp70 functions as a chaperone to prevent such misfolding of the unassembled var1 and, thereby, to ensure efficient subsequent ribosomal assembly.

The requirement of mt-Hsp70 as a chaperone for the mitochondrially translated proteins is supported by the ob-



servation that the assembly of the ATP synthase is also affected in the *sscl* mutants. Both monomers and oligomers of ATPase9 are found in a complex with mt-Hsp70. Furthermore, the formation of a complex of ATPase9 with ATPase6 seems to be adversely affected in the apparent absence of mt-Hsp70 function. As a consequence of this inhibition of ATPase6 assembly, this subunit is rapidly degraded, in contrast to the situation in the wild-type mitochondria, where ATPase6 assembles further towards a functional ATP synthase complex. The 48- and 54-kD complexes described in this report most likely represent assembly intermediates of the ATP synthase. The smaller complex contains ATPase9, whereas the 54-kD complex also contains ATPase6, therefore suggesting that it is a later assembly intermediate.

Other chaperones than mt-Hsp70 may also be involved in the folding and assembly of mitochondrially encoded proteins. In the yeast *mif4* strain, which has a mutated mt-Hsp60 gene, var1 was observed to form aggregates after induction of the temperature-sensitive phenotype (Horwich et al., 1992). Furthermore, a complex has been reported between plant mt-Hsp60 and a protein that is probably a mitochondrial ribosomal protein (Prasad et al., 1990). Mt-Hsp60 was suggested to be involved in the assembly of the ATP synthase in both plant and yeast mitochondria (Prasad et al., 1990; Gray et al., 1990). Together with the results presented here for the mt-Hsp70 mutants, this could indicate a sequential interaction of these chaperones similar to that reported for the assembly of nuclear-encoded mitochondrial proteins (Kang et al., 1990; Manning-Krieg et al., 1991) and for *Escherichia coli* DnaK and GroEL (Langer et al., 1992).

We have described here an important role of the mt-Hsp70 as a chaperone for three mitochondrial gene products. This role comprises (a) maintenance of a soluble state of unassembled proteins; (b) preventing aggregation; and (c) facilitating assembly into larger complexes by selective binding to assembly intermediates. Further functions of mt-Hsp70, such as mediating proteolytic degradation of unassembled proteins and passage of unfolded gene products to mt-Hsp60, seem possible. Quite likely, the reactions studied here represent only part of the chaperone function of mt-Hsp70 for proteins made within mitochondria.

How does mt-Hsp70 fulfill these diverse functions? Although no definite answer can be given to this question, it seems possible that the reversible ATP-dependent binding of mt-Hsp70 to segments of polypeptides lacking a native folded structure is the decisive reaction. By forming such a complex of limited life span, mt-Hsp70 may shift the equilibrium of the various reactions that newly synthesized, unfolded polypeptide chains can undergo. Such reactions include the folding, membrane insertion or translocation, assembly, aggregation, and degradation of these mitochondrially encoded polypeptides.

We are very grateful to Dr. Michael Douglas (University of North Carolina, Chapel Hill, NC) for the kind gift of the antiserum against the yeast  $\alpha$  subunit of the  $F_1$ -ATPase. We would particularly like to thank Drs. Michael Brunner, Douglas M. Cyr, and Thomas Langer for many helpful discussions and advice. We thank also Sandra Weinzierl for excellent technical assistance and Manfred Dünwald for photographic assistance.

This work was supported by grants from the Deutsche Forschungsgemeinschaft Sonderforschungsbereich 184 (Teilprojekt B2) and from the Münchener Medizinische Wochenschrift to R. A. Stuart.

Received for publication 25 April 1994 and in revised form 1 August 1994.

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