# Role of the Chaperonin Cofactor Hsp10 in Protein Folding and Sorting in Yeast Mitochondria

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*Abstract.* Protein folding in mitochondria is mediated by the chaperonin Hsp60, the homologue of *E. coli* GroEL. Mitochondria also contain a homologue of the cochaperonin GroES, called Hsp10, which is a functional regulator of the chaperonin. To define the in vivo role of the co-chaperonin, we have used the genetic and biochemical potential of the yeast *S. cerevisiae*. The *HSP10* gene was cloned and sequenced and temperature-sensitive lethal *hsp10* mutants were generated. Our results identify Hsp10 as an essential component of the mitochondrial protein folding apparatus, participating in various aspects of Hsp60 function. Hsp10 is required for the folding and assembly of proteins imported into the matrix compartment, and is involved in the sorting of certain proteins, such as the Rieske Fe/S protein, passing through the matrix en route to the intermembrane space. The folding of the precursor of cytosolic dihydrofolate reductase (DHFR), imported into mitochondria as a fusion protein, is apparently independent of Hsp10 function consistent with observations made for the chaperonin-mediated folding of DHFR in vitro. The temperature-sensitive mutations in Hsp10 map to a domain (residues 25–40) that corresponds to a previously identified mobile loop region of bacterial GroES and result in a reduced binding affinity of hsp10 for the chaperonin at the non-permissive temperature.

THE folding and assembly of newly synthesized polypeptide chains is mediated by so-called molecular chaperones (Gething and Sambrook, 1992; Hendrick and Hartl, 1993). These proteins interact with non-native polypeptides, preventing unproductive reactions such as aggregation, and provide an environment that permits productive folding in vivo. The members of the Hsp70 and Hsp60 classes of chaperones have been recognized as major players in cellular protein-folding reactions. While the Hsp70s appear to prevent premature folding of incomplete polypeptides during translation and membrane translocation, the Hsp60s mediate the folding of newly synthesized proteins to the native state. Both classes of chaperones can cooperate in a sequential pathway (Langer et al., 1992*a*), which appears to be followed by mitochondrial proteins upon import from the cytosol into the organelles (for review see Hartl et al., 1992). Mitochondrial Hsp70 (Ssc1p of *S. cerevisiae*) binds the extended precursor polypeptides as they emerge from the inner surface of the inner membrane (Kang et al., 1990; Scherer et al., 1990; Gambill et al., 1993). Many or perhaps all imported proteins subsequently interact with Hsp60, the mitochondrial homologue of *E. coli* GroEL, for final folding to the native state (Cheng et al., 1989; Reading et al., 1989; Ostermann et al., 1989; Mizzen et al., 1991; Manning-Krieg et al., 1991).

The Hsp60s (also known as "chaperonins"; Hemmingsen et al., 1988) are large oligomeric ring-complexes. They contain 14 subunits of ~60 kD which are arranged in two stacked heptameric rings. Their basic function is the folding of monomeric polypeptide chains (Ostermann et al., 1989; Martin et al., 1991; Viitanen et al., 1991). The Hsp60 oligomer binds a partially folded protein and releases it in an ATP-dependent reaction resulting in folding (Ostermann et al., 1989; Goloubinoff et al., 1989; Martin et al., 1991, 1993*a*). As shown for GroEL, this process is regulated by the cochaperonin GroES, a single heptameric ring of ~10 kD subunits that forms a complex with GroEL (Viitanen et al., 1990; Langer et al., 1992*b*; Saibil et al., 1993). GroES coordinates the ATPase activity of the GroEL subunits to allow the release of bound polypeptide in a manner productive for folding (Gray and Fersht, 1991; Jackson et al., 1993; Martin et al., 1993*a*, *b*; Todd et al., 1993). However, in vitro studies indicate that this function may not be necessary for the folding of all proteins by GroEL (Laminet et al., 1990; Martin et al., 1991; Viitanen et al., 1991).

Proteins homologous to GroES (Hsp10s) have been identified in mitochondria of different eukaryotic organisms (Lubben et al., 1990; Bertsch et al., 1992; Hartman et al., 1992; Rospert et al., 1993*a*). While the essential role of Hsp60/GroEL in protein folding has been established in vivo (Cheng et al., 1989; Horwich et al., 1993), the conse-

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quences of a loss of Hsp10/GroES function have not yet been investigated in intact cells. We have used the genetic and biochemical potential of *S. cerevisiae* to assess the role of Hsp10 in Hsp60-mediated protein folding in mitochondria. The *HSP10* gene of yeast was cloned and sequenced. Temperature-sensitive lethal *hsp10* mutants were generated, which map to a functionally important loop region in Hsp10 and result in a reduced binding affinity of hsp10 for the chaperonin. The mutant mitochondria show a defect in the folding of several matrix proteins and in the intramitochondrial sorting of the Rieske Fe/S protein. In contrast, the folding of cytosolic dihydrofolate reductase, imported as a mitochondrial fusion protein, appears to be Hsp10-independent.

## Materials and Methods

### Yeast Strains

The Hsp10 protein was isolated from mitochondria of the wild-type strain D273-10B (ATCC 24657). Gene replacement was performed with W303 (*MATα/a, ade2-1, can1-100, his3-11, his3-15, leu2-3, leu2-112, trp1-1, ura3-1*). The haploid P36>S mutant strain (*MATa, ade2-1, can1-100, his3-11, his3-15, leu2-3, leu2-112, trp1-1, ura3-1, Δhsp10::LEU2, pRS413/hsp10*) containing a deletion of the genomic *HSP10* carries the mutagenized *hsp10* (+106 C>T) on the plasmid pRS413 (Sikorski and Hieter, 1989). As an isogenic wild-type control, the same *Δhsp10* strain was used, transformed with *HSP10* on the plasmid pRS316 (Sikorski and Hieter, 1989) (*MATa, ade2-1, can1-100, his3-11, his3-15, leu2-3, leu2-112, trp1-1, ura3-1, Δhsp10::LEU2, pRS316/HSP10*). Expression of Hsp10-β-galactosidase was analyzed in strain UTL-7A (*MATa, leu2-3, trp1-1, ura3-52*).

### Purification of Hsp10

D273-10B cells were grown overnight in YPG medium (1% yeast extract, 2% peptone, 3% glycerol). Mitochondria were isolated as described (Daum et al., 1982; Hartl et al., 1987). The organelles were resuspended in 100 mM Tris-HCl (pH 7.3), 1 mM EDTA, 10 mM KCl (buffer A) and lysed by sonication. After centrifugation (30 min, 100,000 *g*) the supernatant was fractionated on a Superose 6 gel filtration column (Pharmacia LKB Biotechnology, Piscataway, NJ) calibrated in buffer A. Soluble mitochondrial proteins of 10–300 kD were pooled and concentrated to 10 mg/ml. Purified GroEL (25 μg) was added to 2 mg of pooled protein and incubated for 15 min at RT in the absence (buffer A) and presence of MgATP (buffer A, 0.6 mM ATP, 10 mM $MgCl_2$), respectively. Superose 6 gel filtration chromatography was subsequently performed using the corresponding buffer system. Fractions were analyzed by SDS-PAGE according to Schägger and von Jagow (1987). After electroblotting onto nitrocellulose membrane, the Ponceau S-stained Hsp10 polypeptide was excised. In situ tryptic and chymotryptic digestion was performed, the resulting peptides were S-alkylated with 4-vinylpyridine, and separated by narrow bore (2.1 mm) reversed phase HPLC (Ghosh et al., 1990; Tempst et al., 1990). Sequencing was carried out using an optimized 477A instrument (Appl. Biosystems, Inc., Foster City, CA) (Tempst and Riviere, 1989).

### Cloning and Sequencing of HSP10

To amplify *HSP10*, a PCR reaction was performed according to Innis et al. (1990). Yeast genomic DNA of wild-type strain D273-10B, isolated as described (Ausubel et al., 1989), was used as template. The amplified *HSP10* fragment was labeled using the Megaprime DNA labeling system (Amersham Corp., Arlington Heights, IL) and subsequently used to screen a YCp50-based genomic library of *Saccharomyces cerevisiae* (Rose et al., 1987; Ausubel et al., 1989). The localization of the *HSP10* gene within the genomic fragments of the identified plasmids was determined by means of Southern hybridization after restriction digest (Ausubel et al., 1989). The *HSP10* locus surrounding an internal SmaI-restriction site was sequenced with Sequenase (United States Biochemical Corporation, Cleveland, OH) according to the protocol of the manufacturer. Both strands were sequenced after subcloning of restriction fragments into pBluescript SK+ (Stratagene, La Jolla, CA) and by using oligonucleotides against internal sequences as primers. Other recombinant DNA techniques were performed as described by Ausubel et al. (1989).

### Construction of HSP10-lacZ Fusion and Analysis of Expression

A 284-bp HindIII–SmaI fragment comprising the 5′ non-coding region and parts of the open reading frame of *HSP10* was subcloned into YEp356R (Myers et al., 1986). The resulting construct encodes a fusion protein containing amino acids 1-53 of HSP10 and full length β-galactosidase under control of the *HSP10* promoter. The construct was transformed into the *S. cerevisiae* wild-type strain UTL-7A. The transformant was grown overnight at 23°C in SD-medium without uracil (2% Glucose, 0.67% yeast nitrogen base without amino acids supplemented with amino acids according to Ausubel et al. [1989]), and then diluted to an $OD_{600}$ of 0.1 in the same medium. Further incubation was performed at 23 and 37°C. At different time points after the shift, aliquots were taken, cell lysates were prepared, and β-galactosidase activity measured (Ausubel et al., 1989).

### Gene Replacement

A one-step gene replacement was carried out as published (Rothstein, 1983). In brief, a ~830-bp HindIII–ScaI fragment containing *HSP10* was subcloned into pBluescript SK+ (Stratagene). This construct was used as a template in two independent PCR reactions to amplify regions surrounding *HSP10*. Oligonucleotides containing restriction sites were used as primers in these reactions to allow the subcloning of the amplified regions. One PCR product corresponded to nucleotides −28 to −129 of the 5′ non-coding region. The second fragment covered the nucleotides +323 to +716 of the 3′ non-coding region. The PCR products were sequenced and their wild-type character confirmed. Both were subcloned into the integrative vector pRS305 (*LEU2* marker; Sikorski and Hieter, 1989). The resulting construct was linearized with HindIII and transformed into the diploid strain W303 according to the protocol of Gietz and Sugino (1988). Integration into the *HSP10* gene locus was confirmed by Southern hybridization. A *HSP10*/Δhsp10 diploid was then sporulated and tetrads analyzed for spore viability. The diploid strain was also transformed with the 830-bp HindIII–ScaI fragment containing *HSP10* in the *URA3* marker vector pRS316 (WT-*HSP10*). After sporulation and tetrad dissection *Δhsp10* haploids could be isolated carrying a plasmid-derived *HSP10* gene (*Δhsp10*/WT-*HSP10*).

### Screening for a Temperature-Sensitive hsp10 Mutant

A plasmid shuffling experiment (Ausubel et al., 1989) was performed to identify temperature-sensitive *hsp10* mutants after chemical mutagenesis of the isolated *HSP10*. Hydroxylamine mutagenesis of the plasmid WT-*HSP10* was carried out as described by Busby et al. (1982) including incubation at 75°C for 30 min. The mutagenized gene was subcloned as a 830-bp HindIII–ScaI fragment into the *HIS3*-containing vector pRS413. 3,500 independent bacterial clones were obtained and their plasmid DNA prepared. The haploid yeast strain *Δhsp10*/WT-*HSP10* was transformed with this mutagenized *hsp10* library. Yeast transformants were replica-plated onto medium containing 5-fluoroorotic acid and uracil to induce a loss of the plasmid WT-*HSP10* and incubated at 23°C (according to Ausubel et al., 1989). The resulting haploids containing exclusively the mutagenized form of the *HSP10* gene were then screened for growth at 23°C and 37°C on SD-medium without histidine and leucine.

The G32>D point mutation was introduced into the *HSP10* gene by primer-mediated mutagenesis after the protocol of Innis et al. (1990). The mutagenized gene was subcloned into the vector pRS413. Plasmid shuffling and analysis of growth behavior was performed as described above.

### Import of Precursor Proteins into Isolated Mitochondria

*Δhsp10* strains transformed with the wild-type *HSP10* gene (WT-*HSP10*) and the temperature sensitive allele (ts-*hsp10*), respectively, were grown overnight at 23°C in YPGal medium (1% yeast extract, 2% peptone, 2% galactose) to an $OD_{600}$ of 1.0. Mitochondria were isolated essentially as described above, but zymolyase treatment was performed at 25°C. After differential centrifugation the organelles were resuspended in SEM (250 mM sucrose, 1 mM EDTA, and 10 mM MOPS, pH 7.2) to a protein concentration of 5 mg/ml.

The genes of mitochondrial precursors were transcribed from pGEM plasmids using SP6 polymerase according to the manufacturer (Promega Corp., Madison, WI). Precursor proteins were synthesized in the presence of [$^{35}$S]methionine in reticulocyte lysate (Promega). After translation, postribosomal supernatants were prepared according to Zimmermann and

Neupert (1980). Import reactions contained 10–20% postribosomal supernatants, 0.4 mg/ml mitochondria, 2 mM ATP, 2 mM NADH, and 4 mM $MgCl_2$, in BSA-buffer (3% BSA, 220 mM sucrose, 80 mM KCl, and 10 mM MOPS, pH 7.2). Inhibition of the membrane potential and protease treatment of mitochondria were performed as published (Hartl et al., 1986). To test for the aggregation of imported proteins, mitochondria were lysed in 1 mM EDTA, 40 mM NaCl, and 20 mM MOPS, pH 7.2, containing 1% Triton X-100 and subsequently centrifuged for 20 min at 30,000 *g*.

Assembly of rat ornithine transcarbamylase was analyzed by binding the protein to δ-N-(phosphono-acetyl)-L-ornithine (PALO)[1]-Sepharose (Hoogenraad et al., 1980). After in vitro import of pre-ornithine transcarbamylase (pre-OTC) at 37°C for 25 min, mitochondria were proteinase K treated as described above. Reisolated mitochondria (150 μg) were lysed in 500 μl 20 mM Hepes, pH 7.4, 1% Triton X-100, and centrifuged at 15,000 *g* for 15 min. The supernatant was applied to a 200-μl PALO-column equilibrated in 10 mM Hepes, pH 7.4. The column was washed with 2 ml of the same buffer and 2 ml of 10 mM Hepes, pH 7.4, 40 mM KCl. Assembled OTC was specifically eluted with 25 μM carbamylphosphate, 10 mM Hepes, pH 7.4.

Radiolabeled pre-Su9-dihydrofolate reductase (DHFR) (containing residues 1-69 of *N. crassa* ATPase subunit 9 fused to full length mouse DHFR; Ostermann et al., 1989) synthesized in reticulocyte lysate was precipitated by addition of ammonium sulphate to a final concentration of 40% for 30 min at 0°C. The precipitate was collected by centrifugation at 15,000 *g* for 10 min, and then dissolved in 8 M urea, 20 mM MOPS pH 7.2, 60 mM DTT. The denatured precursor was 30-fold diluted into import reactions according to Koll et al. (1992).

### *In Vitro Transcription/Translation of HSP10 and ts-hsp10*

A BamHI restriction site was introduced upstream of the *HSP10* open reading frame using PCR with the plasmid WT-*HSP10* as a template. The obtained PCR product was sequenced to confirm its wild-type character and a BamHI–SalI fragment containing *HSP10* subcloned into pGEM (Promega). In a similar way the ts-allele was amplified using the plasmid ts-*hsp10* as the template and also subcloned into pGEM. Both genes were transcribed and translated as described above and postribosomal supernatants were used for in vitro import experiments into isolated mitochondria.

### *Miscellaneous*

GroEL was purified from a *groE*-overproducing strain of *E. coli* harboring the plasmid pOF39 (Fayet et al., 1986; Martin et al., 1991). Non-denaturing gel electrophoresis (native PAGE) was performed using 3–10% polyacrylamide gradient gels in 80 mM MOPS, pH 7.2 which were run for 16 h at 120 V. To analyze complex formation between GroEL and Hsp10 by native PAGE, polyacrylamide gels and running buffer included 1 mM $MgCl_2$ and 0.2 mM ATP.

## *Results*

### *Purification of Hsp10 from Yeast Mitochondria*

The GroES homologues in rat liver mitochondria (Hsp10) and pea chloroplasts are able to form stable complexes with bacterial GroEL in the presence of MgATP (Lubben et al., 1990; Bertsch et al., 1992). We used this property to identify the mitochondrial Hsp10 protein of *S. cerevisiae*. Soluble matrix proteins of 10–300 kD were prepared from yeast mitochondria and incubated with purified GroEL in the presence of MgATP followed by gel filtration chromatography on a Superose 6 column. An ~12-kD polypeptide cofractionated with the 800-kD GroEL as detected by SDS-PAGE (Fig. 1). This cofractionation was not observed in the absence of MgATP (Fig. 1). The 12-kD polypeptide was transferred onto

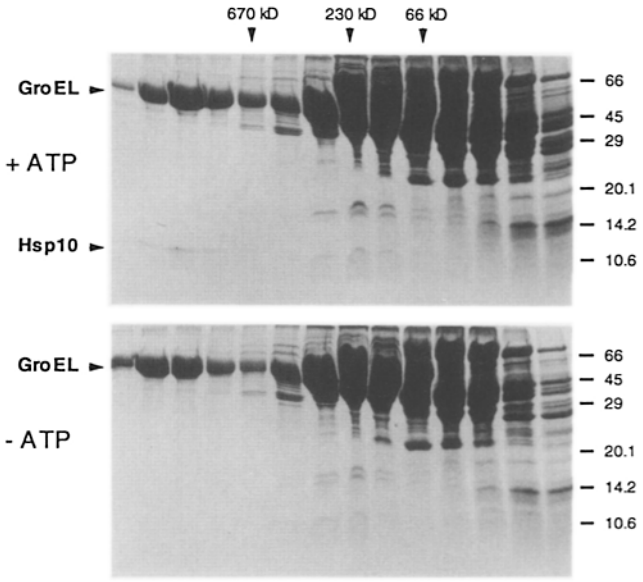


*Figure 1.* Purification of mitochondrial Hsp10 from *Saccharomyces cerevisiae* based on MgATP-dependent complex formation with bacterial GroEL. Size-fractionated soluble mitochondrial proteins of 10–300 kD (2 mg protein) were incubated with 25 μg GroEL in the presence and absence of MgATP and subsequently fractionated on a Superose 6 gel filtration column. In the presence of MgATP, the 12-kD Hsp10 protein of yeast cofractionates with the 800-kD GroEL complex.

a nitrocellulose membrane and subjected to tryptic and chymotryptic digestion. Three HPLC purified peptides were sequenced (Fig. 2 *A*). All three peptides showed significant sequence homology to bacterial GroES and rat liver Hsp10 (Fig. 2 *B*).

The sequence homology as well as the ability to form a complex with GroEL in the presence of MgATP identified the purified 12-kD polypeptide as the yeast mitochondrial homologue of GroES, henceforth termed yeast Hsp10. When the isolated GroEL-Hsp10 complex was subjected to a second gel filtration chromatography in the absence of MgATP, Hsp10 eluted at a size of ~80 kD (data not shown), which is characteristic of the homo-oligomeric complexes formed by GroES and its homologues (Viitanen et al., 1990; Langer et al., 1992*b*; Hartman et al., 1992).

### *Cloning and Sequencing of the Yeast HSP10 Gene*

Based on the obtained peptide sequences, a set of degenerate oligonucleotide primers was designed and used in a PCR reaction containing genomic DNA of *S. cerevisiae*. An internal ~120-bp fragment of the *HSP10* gene could be amplified and was subsequently radiolabeled to screen a YCp50 yeast genomic library (Rose et al., 1987). Three different but overlapping 10–15 kb genomic DNA inserts were identified which contained the *HSP10* gene. Sequence analysis of a 534-bp region surrounding a unique internal SmaI restriction site revealed an open reading frame of 318 bp encoding a putative protein of 106 amino acids with a calculated molecular mass of 11,374 D (Fig. 2 *A*). The encoded protein contained the peptide sequences of the purified 12-kD Hsp10 protein (Fig. 2 *A*). The complete Hsp10 sequence shows 36.5% identity to *E. coli* GroES and 43.6% identity to rat

1. *Abbreviations used in this paper*: DHFR, dihydrofolate reductase; HSE, heat shock responsive element; MPP, matrix processing protease; PALO, δ-N-(phosphono-acetyl)-L-ornithine; pre-OTC, pre-ornithine transcarbamylase.

**A**

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                                        HindIII
-126                                    AAGCTTGATTATGTATAAAATTAGTCAAAACGCTTATACAGTACAAA

 -79 CTTATTATGTGCTAGGTTTAAAATAATTTCTGATAGAAGCCATTGATCGAAAATATATTGAATCTACAGAAAAAAAAGA

   1 Met Ser Thr Leu Leu Lys Ser Ala Lys Ser Ile Val Pro Leu Met Asp Arg Val Leu Val
  +1 ATG TCC ACC CTT TTG AAG TCT GCT AAA TCT ATC GTT CCA TTG ATG GAC CGT GTC CTT GTC

  21 Gln Arg Ile Lys Ala Gln Ala Lys Thr Ala Ser Gly Leu Tyr Leu Pro Glu Lys Asn Val
 +61 CAA AGA ATC AAG GCA CAA GCA AAG ACA GCA TCC GGG TTG TAT TTA CCT GAA AAG AAC GTG

                                                SmaI
  41 Glu Lys Leu Asn Gln Ala Glu Val Val Ala Val Gly Pro Gly Phe Thr Asp Ala Asn Gly
+121 GAG AAG TTA AAC CAA GCT GAA GTT GTT GCC GTA GGC CCG GGC TTT ACT GAT GCT AAT GGT

  61 Asn Lys Val Val Pro Gln Val Lys Val Gly Asp Gln Val Leu Ile Pro Gln Phe Gly Gly
+181 AAT AAG GTT GTT CCT CAA GTT AAA GTT GGT GAC CAA GTT TTG ATT CCA CAG TTT GGT GGT

  81 Ser Thr Ile Lys Leu Gly Asn Asp Asp Glu Val Ile Leu Phe Arg Asp Ala Glu Ile Leu
+241 TCT ACC ATT AAA TTG GGT AAC GAC GAT GAA GTT ATT CTT TTC AGG GAC GCT GAA ATC CTG

 101 Ala Lys Ile Ala Lys Asp TER
+301 GCT AAG ATT GCC AAG GAC TAA GAATGATGTCTTCTTCAACAGAGATTTTATATACATACTCTATATGTATGT

+373 ACCTGTAAATAGGCCATTATGTATGACAGGAAAAAA
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**B**

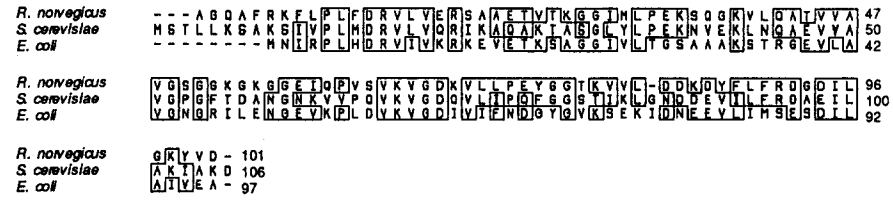



*Figure 2.* *(A)* Nucleotide sequence of the *HSP10* gene of *Saccharomyces cerevisiae* and deduced amino acid sequence. The putative heat shock responsive elements (HSEs) in the 5′ non-coding region are underlined. Peptide sequences obtained from purified Hsp10 are shown double-underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession number X75754. *(B)* Sequence alignment of yeast Hsp10 with rat liver Hsp10 (Hartman et al., 1993) and *E. coli* GroES (Hemmingsen et al., 1988) created using PILEUP (Genetics computer group, Wisconsin). Boxes highlight identical or similar residues.

liver Hsp10. A sequence alignment of the three proteins is presented in Fig. 2 *B*.

The $NH_2$-terminal 30 residues of yeast Hsp10 have a positive net charge of +6 and contain five hydroxylated amino acids. Structure prediction analysis indicates a mostly $\alpha$-helical conformation for this region. These are the hallmarks of mitochondrial targeting sequences that direct proteins into the matrix (Hartl et al., 1989). However, a consensus site for proteolytic cleavage of the putative targeting sequence by the mitochondrial processing enzyme could not be identified. Consistent with this, we did not detect proteolytic cleavage of Hsp10 upon import into isolated mitochondria (not shown).

### *Essential Requirement of Hsp10 for Cell Growth*

To investigate whether the *HSP10* gene is essential for growth of *S. cerevisiae*, a one-step gene replacement was performed. This strategy generated a diploid strain with one wild-type copy of the *HSP10* gene and one copy replaced by the *LEU2* marker. The replacement was confirmed by Southern hybridization. Upon sporulation and incubation of dissected tetrads at 30°C, a 2:2 segregation of viable:nonviable spores was observed, suggesting that *HSP10* is an essential gene at normal growth temperature. The *HSP10/Δhsp10* diploid was also transformed with a wild-type copy of the *HSP10* gene on a *URA3* containing plasmid before sporulation. *LEU2/URA3* haploids were isolated which were unable to grow at temperatures between 23°C and 37°C after counterselection against the plasmid borne wild-type copy of *HSP10* on 5-fluoroorotic acid containing medium (Boeke et al., 1984). This confirmed the essential nature of the *HSP10* gene product.

### *Induction of HSP10 Expression upon Temperature Shift*

The 5′ non-coding region of the *HSP10* gene contains a putative heat shock responsive element (HSE). Yeast HSEs are typically composed of three to five GAA or TTC modules separated from each other by two nucleotides (Boorstein and Craig, 1990). Five such modules are present upstream of the *HSP10* gene (Fig. 2 *A*). However, they are separated by 5–10 nucleotides. To test whether *Hsp10* expression is heat inducible, an *HSP10-lacZ* fusion was constructed which encodes the amino terminal 53 residues of Hsp10 followed by $\beta$-galactosidase under control of the *HSP10* promoter. After transformation of this construct into a yeast wild-type strain, cells were grown overnight at 23°C, and then maintained at 23°C or transferred to 37°C. Upon shift to the elevated temperature, a 2.3-fold induction of $\beta$-galactosidase activity was observed (Fig. 3). The enzymatic activity of the reporter protein remained increased for up to 6 h after temperature shift. Similar observations were made upon shift to 39°C (data not shown). These results suggest that the transcription of *HSP10* is upregulated upon a shift to increased growth temperatures. The ~12-kD gene product can be classified as a novel heat shock protein of yeast.

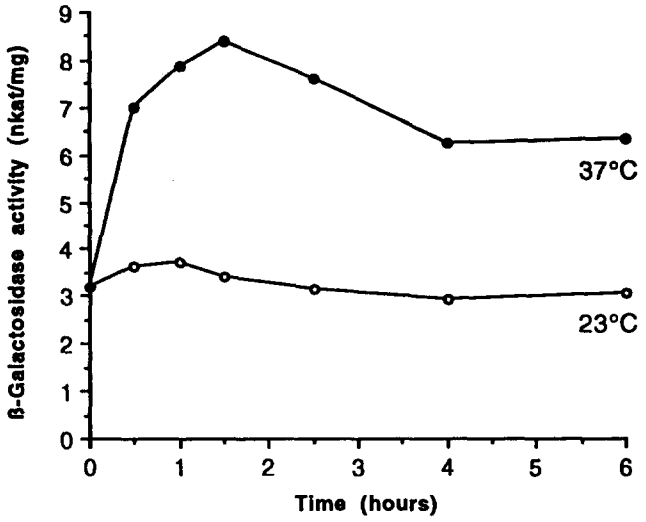
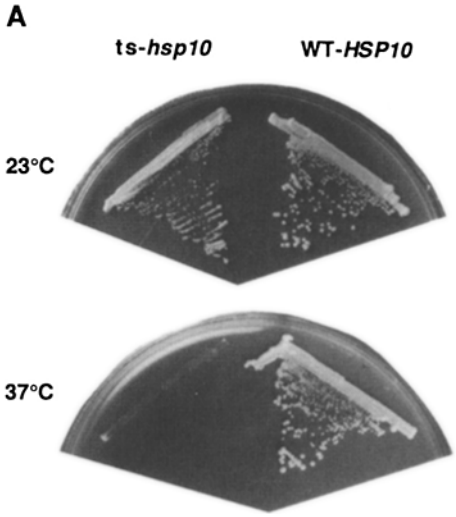



*Figure 3.* Induction of expression of a Hspl0-β-galactosidase fusion protein upon heat shock. Wild-type yeast cells carrying a *HSP10-lacZ* fusion gene under control of the *HSP10* promoter were grown overnight at 23°C in SD-medium. Cells were diluted to an $OD_{600}$ of 0.1 in the same medium, further incubated at 23°C and 37°C, and β-galactosidase activities were measured. Transfer to the increased growth temperature resulted in a 2.3-fold induction of the enzymatic activity of the reporter protein.




B

| | | |
|---|---|---|
| *E.coli* | 17 | VETKSAGGIVLTGSAA 32 |
| *M.tuberculosis* | 20 | AETTTASGLVIPDTAK 35 |
| *P.aeruginosa* | 17 | EETKTAGGIVLPGSAA 32 |
| *L.micdadei* | 17 | EERTTAGGIVIPDSAT 32 |
| *C.burnetti* | 17 | EERTSAGGIVIPDSAA 32 |
| *Rattus norvegicus* | 23 | AETVTKGGIMLPEKSQ 38 |
| *S.cerevisiae* | 25 | AQAKTASGLYLPEKNV 40 |

*Figure 4.* The P36>S *hspl0* mutant shows a temperature-sensitive phenotype. (*A*) A Δ*hspl0* haploid strain carrying the temperature-sensitive *hspl0* allele on a single copy plasmid (*ts-hspl0*) grows normally at 23°C, but does not grow at 37°C. As a control, the deleted strain transformed with a wild-type copy of *HSP10* on a single copy plasmid is shown (*WT-HSP10*). Growth behavior was analyzed on 2% glucose SD-medium. (*B*) Sequence comparison of the mobile loop domain of *E. coli* GroES (Landry et al., 1993) with the homologous region of GroES-like proteins. The ts-mutation of yeast Hspl0 alters the conserved proline residue at position 36 to serine (*double-underlined*). A change of glycine 32 (*underlined*) to aspartic acid also results in a temperature-sensitive phenotype (see text).

## *Isolation of Temperature-sensitive Alleles of HSP10*

To examine the consequences of a loss of Hspl0 function for mitochondrial protein folding, a screen for temperature-sensitive (ts) *hspl0* mutants was conducted. After hydroxylamine-treatment of the plasmid WT-*HSP10*, the *HSP10* gene was subcloned into a *HIS3* single copy vector. The resulting library of mutagenized *hspl0* was subsequently transformed into the haploid Δ*hspl0* deletion strain, which carried a wild-type copy of the gene on a *URA3* plasmid. Double transformants were counterselected at 23°C against the presence of the *URA3* plasmid and the clones obtained, containing exclusively the mutagenized *hspl0* gene, were analyzed for growth at 23°C and 37°C. Among 2,500 transformants, two clones were identified that were unable to grow at the elevated temperature (one shown in Fig. 4 *A*). The ts-phenotype was not observed when these clones were incubated at both temperatures before counterselection against the wild-type copy of the *HSP10* gene, indicating that the mutagenized plasmid is responsible for the observed ts-phenotype. Sequence analysis of the mutated *hspl0* gene of both clones revealed a single C>T transition at position +106, changing residue 36 of Hspl0 from proline to serine (Fig. 4 *B*). The proline residue at this position is highly conserved in several GroES homologues with the exception of *E. coli* GroES (Fig. 4 *B*).

Interestingly, proline 36 resides in a region of yeast Hspl0 (residues 25–40) that corresponds to a "mobile loop" domain of GroES extending between residues 17 and 32 (Landry et al., 1993) (Fig. 4 *B*). In GroES a different point mutation within this region, glycine 24 to aspartic acid, results in a temperature-sensitive growth phenotype in *E. coli* (Landry et al., 1993). Glycine 24 is conserved in yeast Hspl0 (G32; Fig. 4 *B*). When the G32>D mutation was introduced into *HSP10*, the resulting yeast mutant strain was unable to grow at 37°C and showed an eightfold reduced growth rate at 23°C. These results indicate that the function of Hspl0 is required for growth over a wide temperature range and confirm the importance of the mobile loop region in the Hspl0/GroES cochaperonins.

## *Mutant hspl0 Has a Reduced Binding Affinity for the Chaperonin*

It seemed possible that the P36>S mutant hspl0 was compromised in its ability to form a complex with Hsp60. To test this hypothesis, wild-type Hspl0 and mutant hspl0 were synthesized as radiolabeled proteins by in vitro transcription/translation and imported at the permissive temperature into wild-type and mutant mitochondria, respectively. Under these conditions both proteins assemble to the 80-kD Hspl0 oligomer with similar efficiency, as determined by gel filtration chromatography (data not shown). This assembly most likely occurs by subunit exchange with preexistent Hspl0 (see below). After in vitro import, mitochondrial extracts containing radiolabeled mutant and wild-type Hspl0

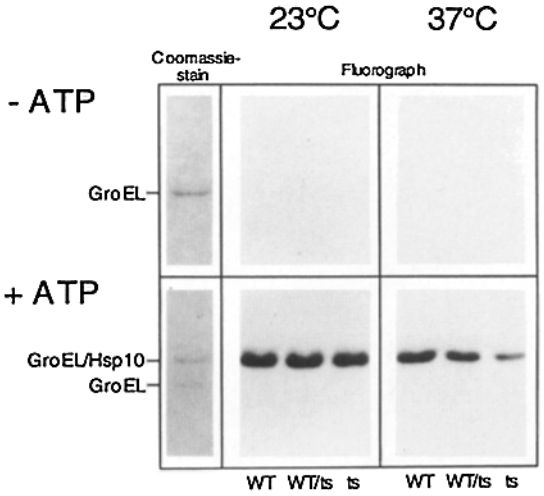



*Figure 5.* P36>S hspl0 binds to GroEL with reduced affinity at the non-permissive temperature. Radiolabeled wild-type and mutant Hspl0-oligomer were obtained after in vitro transcription/translation and subsequent import at 23°C for 30 min into mitochondria isolated from wild-type and mutant cells grown at 23°C. Wild-type Hspl0 was imported into wild-type (*WT*) and mutant organelles (*WT/ts*), and mutant hspl0 into mutant mitochondria (*ts*). Import was stopped by addition of 1 μM valinomycin and incubation was continued for 10 min. The reactions were twofold diluted with ice-cold BSA-buffer (see Materials and Methods), and treated with 20 μg/ml proteinase K for 10 min at 0°C. Digestion was stopped by addition of 1 mM PMSF, and mitochondria were reisolated by centrifugation. The organelles were resuspended in SEM buffer/10 mM KCl to a protein concentration of 0.25 mg/ml, and divided into aliquots containing equal amounts of imported Hspl0. Mitochondria were lysed by addition of the non-ionic detergent Genapol (0.1% final concentration) and lysates were incubated for 10 min at 23°C and 37°C in the presence of 20 U/ml apyrase, 5 mM CDTA (−ATP) or 2 mM ATP, 10 mM $MgCl_2$ (+ATP). Purified GroEL was added to all reactions to a concentration of 10 μg/ml. After incubation for 2 min at the indicated temperatures, native PAGE was performed in the absence and presence of MgATP in the gel buffer at 23°C and 37°C, respectively. ATP-dependent complex formation between radiolabeled Hspl0 and GroEL was observed. The complex migrates with a reduced mobility relative to GroEL alone.

oligomer were prepared and the ability of Hspl0 to associate with the chaperonin was analyzed. Based on the observation that the interaction of yeast mitochondrial Hspl0 with GroEL is fully functional (see above; Rospert et al., 1993*a*), purified GroEL was added to the mitochondrial lysates and the formation of the Hspl0-GroEL complex was analyzed by native polyacrylamide gel electrophoresis (native PAGE). When the extract was depleted of ATP by apyrase treatment, no interaction between Hspl0 and GroEL was detectable (Fig. 5). In contrast, incubation with purified GroEL in the presence of MgATP resulted in the highly efficient formation of a radiolabeled complex that migrated on native PAGE with a reduced mobility compared to GroEL. Such a mobility shift of GroEL occurs typically upon complex formation with GroES (Langer et al., 1992*b*). The MgATP dependence of the interaction indicates that the cochaperonin was bound specifically, since unfolded substrate protein would be released in the presence of MgATP. In the absence of GroEL, ATP-dependent complex formation between Hspl0 and endogenous Hsp60 was observed (not shown). However, this interaction was less efficient than binding of Hspl0 to added GroEL, due to the low protein concentration of the mitochondrial lysates.

When the incubation with GroEL and native PAGE were performed at 23°C, similar amounts of mutant and wild-type Hspl0 bound to the chaperonin (Fig. 5). Wild-type Hspl0 also interacted normally with GroEL at 37°C. In contrast, the amount of P36>S hspl0 that bound to GroEL at 37°C was diminished by 80%. Size exclusion chromatography at 37°C demonstrated that the mutant hspl0 oligomer does not dissociate at the elevated temperature (not shown). These data demonstrate that P36>S hspl0 has a reduced affinity for the chaperonin under non-permissive conditions. This decrease in hspl0 binding to GroEL was not observed when mutant organelles were first treated at 37°C before lysis but the binding analysis was carried out at 23°C (Fig. 5), indicating that the mutant phenotype is reversible.

Interestingly, when radiolabeled wild-type Hspl0 was imported into mutant mitochondria under permissive conditions, the resulting Hspl0 oligomer showed an intermediate level of binding to GroEL at 37°C (Fig. 5). This suggests that the newly imported, radiolabeled protein can assemble into the oligomer by subunit exchange with preexisting mutant hspl0.

### *Requirement of Hspl0 for Folding of Imported Proteins*

The temperature-sensitive yeast strain carrying the P36>S mutation in *hspl0* was used to investigate the role of Hspl0 in the folding of precursor proteins imported into isolated mitochondria. The organelles were isolated from mutant cells grown at 23°C. Under these conditions the temperature-sensitive strain exhibited normal growth (Fig. 4 *A*). The mutant phenotype was then induced by a 10-min incubation of the isolated mitochondria at 37°C, based on our observation that the elevated temperature affected the conformation of the preexistent mutant protein. This allowed us to study the consequences of a loss of Hspl0 function *in organello* under well defined conditions, minimizing possible secondary effects of the mutation. A Δ*hspl0* deletion strain transformed with a wild-type copy of the *HSP10* gene was used as wild-type control. In contrast to the P36>S mutation, the G32>D mutant strain did not permit the isolation of import competent mitochondria.

In a first series of experiments the import and folding of the α-subunit of the yeast mitochondrial matrix processing protease (α-MPP, 53 kD) was analyzed. After import, α-MPP has to fold and assemble with the β-MPP subunit (Yang et al., 1988). α-MPP was synthesized as a radiolabeled precursor in reticulocyte lysate. Import was performed either at 23°C or at 37°C after preincubation of the mitochondria at the respective temperature. To assess incorrect folding of α-MPP, the amount of Triton X-100 insoluble material was determined after lysis of the organelles and high speed centrifugation. Similar amounts of protease protected, mature-sized α-MPP were detected in mitochondria of wild-type and mutant strain (Fig. 6 *A*), excluding a defect in membrane translocation and proteolytic processing of precursor proteins. Strikingly, 60–70% of α-MPP imported into mutant mitochondria at the non-permissive temperature was recovered in detergent-insoluble aggregates, whereas the protein

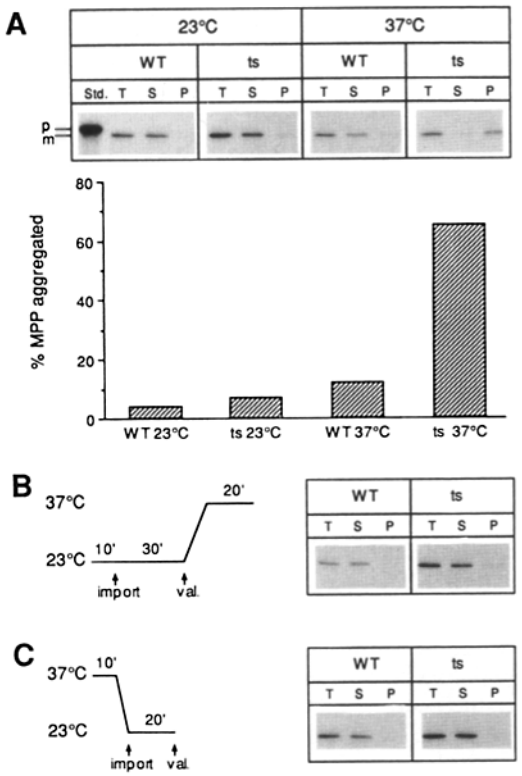



*Figure 6.* In vitro import of pre-α-MPP into isolated mitochondria of the P36>S *hspl0* mutant (*ts*) and the corresponding wild-type strain (*WT*). (*A*) Import of pre-α-MPP into mutant mitochondria at the non-permissive temperature results in the formation of detergent insoluble aggregates. Import reactions containing mitochondria (0.4 mg/ml) isolated from cells grown at 23°C were preincubated at 23°C and 37°C for 10 min. Subsequently, reticulocyte lysate containing the radiolabeled precursor of yeast α-MPP (*Std.*) was added and further incubated at the indicated temperatures. The import reactions were stopped by dilution into the same volume of ice-cold BSA-buffer (containing 40 U/ml apyrase) and addition of 1 μM valinomycin. All reactions were treated with proteinase K (20 μg/ml) for 10 min at 0°C. Protease treatment was inhibited by addition of 1 mM PMSF, and mitochondria were reisolated by centrifugation. Mitochondria were solubilized in Triton buffer (20 mM MOPS, pH 7.2, 40 mM NaCl, 1 mM EDTA, 40 U/ml apyrase, 1% Triton X-100). An aliquot was taken as the total protein sample (*T*). The remainder was separated into supernatant (*S*) and pellet fractions (*P*) by centrifugation at 30,000 *g* for 20 min. All reactions were analyzed by SDS-PAGE and fluorography. Amounts of α-MPP were quantified using a Phosphorimager system (Molecular Dynamics, Inc., Sunnyvale, CA). (*B*) Incubation of mutant mitochondria at the non-permissive temperature after import at 23°C does not result in the aggregation of α-MPP. Import was performed as described above at 23°C for 30 min. After addition of 1 μM valinomycin, the reactions were shifted to 37°C and incubated for 20 min. Further analysis was performed as described above. (*C*) Aggregation was not observed when isolated mitochondria were pretreated at 37°C for 10 min and import subsequently performed at 23°C.

remained largely soluble in wild-type mitochondria at 37°C and upon import into mutant mitochondria at 23°C (Fig. 6 *A*). Notably, Hsp60 was fully soluble in the hspl0 mutant (not shown), indicating that α-MPP did not remain associated with the chaperonin at the non-permissive temperature. These results demonstrate that the defect in Hspl0

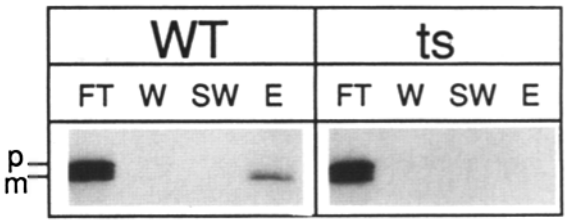



*Figure 7.* Inactivation of Hspl0 affects the assembly of rat OTC in isolated yeast mitochondria. Radiolabeled precursor of rat ornithine transcarbamylase was imported into wild-type (*WT*) and mutants (*ts*) mitochondria at 37°C for 25 min. Mitochondria were proteinase K treated, reisolated, and lysed in 20 mM Hepes, pH 7.4, 1% Triton X-100. After centrifugation at 15,000 *g* for 15 min, the soluble fraction was applied to a PALO-resin, and purification of assembled, trimeric OTC was performed as described in Materials and Methods. *FT* corresponds to 10% of the flow through fraction. The resin was washed with 10 mM Hepes, pH 7.4 (*W*) and 10 mM Hepes, pH 7.4, 40 mM KCl (*SW*). Assembled, mature OTC was eluted with 25 mM carbamylphosphate, 10 mM Hepes, pH 7.4 (*E*). In addition to the precursor and mature forms, intermediate-sized OTC is visible in the flow through fraction.

causes misfolding of newly imported α-MPP. When import was performed at 23°C after a preincubation of the isolated mutant mitochondria at 37°C, no aggregation of α-MPP was observed (Fig. 6 *C*). This is consistent with the finding that the reduction in binding of P36>S hspl0 to the chaperonin at 37°C is fully reversible (see above). It cannot be excluded, however, that the folding of α-MPP requires Hspl0 function only at the elevated temperature.

Is the function of Hspl0 also required for the maintenance of the folded state of α-MPP at 37°C? Precursor of α-MPP was imported at 23°C for 30 min. After inhibition of further import by adding the uncoupler valinomycin, the samples were shifted to 37°C and incubated at the elevated temperature for 20 min. Under these conditions the imported α-MPP remained soluble in both mutant and wild-type mitochondria (Fig. 6 *B*). This shows that the inactivation of Hspl0 at 37°C specifically affects the de novo folding pathway of α-MPP.

We also analyzed the assembly of rat mitochondrial OTC after import into isolated mitochondria. Pre-OTC, imported into yeast mitochondria, has been shown to assemble into the catalytically active trimer in an Hsp60-dependent manner (Cheng et al., 1989). Upon import at 37°C, similar amounts of mature-sized, soluble OTC were detected in wild-type and mutant mitochondria (Fig. 7). Proteolytic processing of pre-OTC in yeast mitochondria was relatively inefficient, but this was independent of the mutant phenotype. Correct folding and assembly of mature OTC was assessed by chromatography on PALO columns. It has been shown that only trimeric OTC binds to this transition-state substrate analogue (Cheng et al., 1989). In wild-type mitochondria, ~5% of the mature protein assembled to the trimer, based on its specific elution with carbamylphosphate (Fig. 7). The low efficiency of assembly is most likely due to the fact that only pmole amounts of precursor protein are synthesized in reticulocyte lysate and no endogenous OTC is present in yeast mitochondria. In contrast, trimeric OTC was not detectable after import in the *hspl0* mutant organelles under non-permissive conditions (Fig. 7), demonstrating the essential role of Hspl0 for the assembly of OTC.

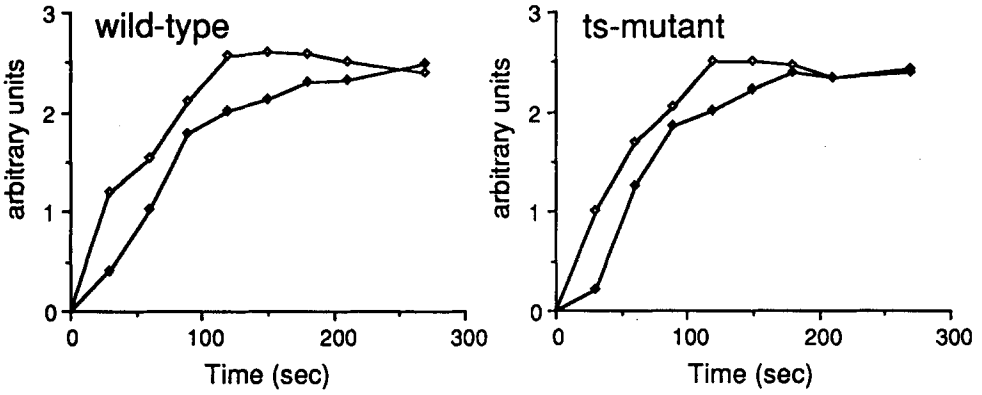



*Figure 8.* Kinetics of import and folding urea-denatured Su9-DHFR in isolated mitochondria at 37°C. Denatured radiolabeled pre-Su9-DHFR was diluted 30-fold into import reactions (0.5 mg/ml mitochondria, 250 mM sucrose, 10 mM MOPS pH 7.2, 80 mM KCl, 5 mM $MgCl_2$, 2 mM NADH, 2 mM ATP) that had been preincubated for 10 min at 37°C. At the time points indicated, aliquots were removed and diluted twofold into ice cold SEM buffer. Half of each sample was treated with 20 μg/ml proteinase K for 10 min on ice to assess import (*open symbols*). The other half was lysed by addition of digitonin (0.3% final concentration), 100 mM NaCl, 10 mM CDTA, and protease treatment was performed as above to monitor folding of DHFR (*closed symbols*). After inhibition of the protease with 1 mM PMSF, samples were analyzed by SDS-PAGE and Phosphorimager quantification was performed.

Certain small proteins, such as DHFR, do not depend on the function of the cochaperonin during Hsp60/GroEL-mediated refolding in vitro (Martin et al., 1991; Viitanen et al., 1991). The requirement of Hsp60 for DHFR folding has been demonstrated (Ostermann et al., 1989; Martin et al., 1992). We analyzed the dependence of this reaction on the cochaperonin *in organello*. Su9-DHFR, a fusion protein carrying the presequence of subunit 9 of the $F_0$-ATPase, was imported from reticulocyte lysate into mutant mitochondria at 37°C. The protein was processed to the mature size and remained soluble (data not shown). To analyze whether inactivation of Hsp10 affected the kinetics of Su9-DHFR folding, the fusion protein was denatured in 8 M urea and rapidly diluted into import reactions at 37°C. Under these conditions, membrane translocation of the precursor was complete within 120 s (Fig. 8), and subsequent folding was monitored by measuring the acquisition of protease resistance of DHFR after lysing the organelles (Ostermann et al., 1989). The P36>S hsp10 mutation had no detectable effect on the kinetics of DHFR folding (Fig. 8). Since the mutant hsp10 protein appears to retain a residual capacity to bind to the chaperonin at the non-permissive temperature, the participation of Hsp10 in DHFR folding cannot, however, be ruled out completely.

### *Role of Hsp10 in Intramitochondrial Protein Sorting*

Hsp60 has also been implicated in the sorting of proteins from the matrix to the inner membrane and intermembrane space (Hartl and Neupert, 1990). For example, the Rieske Fe/S protein is completely translocated into the matrix, where it is proteolytically processed in two steps before export across the inner membrane (Hartl et al., 1986; Cheng et al., 1989; Isaya et al., 1992). Presumably, Hsp60 maintains the protein in a non-aggregated conformation competent for the second translocation step. We tested whether this function of Hsp60 is dependent on Hsp10. Upon import at 23°C, most of the Fe/S precursor was fully processed in both mutant and wild-type mitochondria and was recovered in the Triton X-100 soluble organelle fraction (Fig. 9). When import into wild-type mitochondria was performed at 37°C, the efficiency of processing was slightly reduced and a small amount of protein was Triton insoluble. Significantly, upon translocation into mutant organelles under non-permissive conditions, the processing intermediate of the Fe/S protein accumulated (Fig. 9). Most of this protein was in the detergent insoluble sediment, reflecting misfolding and aggregation of Fe/S in the matrix compartment before export. Fe/S protein imported at 23°C did not aggregate during incubation of mutant mitochondria at the elevated temperature (data not shown). These results indicate the essential role of Hsp10 in the assembly pathway of newly imported Fe/S protein.

The biogenesis of a fusion protein containing the amino-terminal 167 residues of precytochrome $b_2$ followed by the complete sequence of mouse dihydrofolate reductase ($b_2$ (167)-DHFR) was also found to be affected in the P36>S

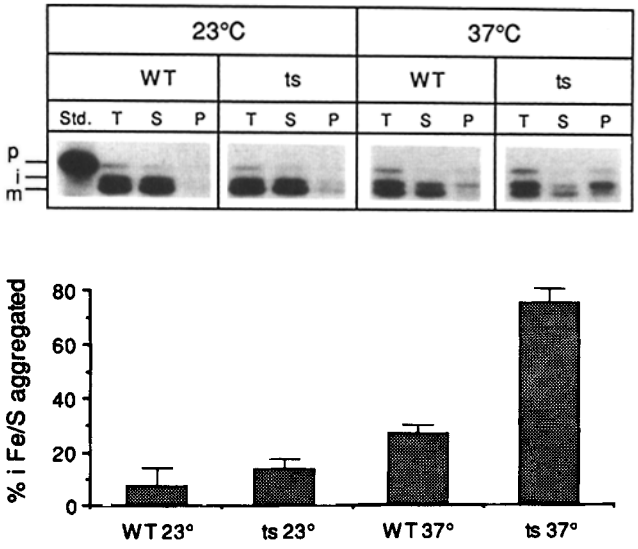



*Figure 9.* The P35>S mutation of hsp10 affects the assembly pathway of the yeast iron/sulfur protein (*Fe/S*). In vitro import of radiolabeled precursor of the Fe/S protein into isolated wild-type (*WT*) and mutant (*ts*) mitochondria at the permissive (23°C) and non-remissive temperature (37°C). Experimental conditions were as described in the legend to Fig. 5. The lower panel shows the mean value and standard deviation obtained in five independent experiments.

*hspl0* mutant. During its sorting to the intermembrane space precytochrome $b_2$ is processed in two steps (Van Loon et al., 1986; Hartl et al., 1987). The $NH_2$-terminal matrix-targeting signal is removed by the MPP, resulting in an intermediate-sized form carrying a bacterial type export signal (Hartl and Neupert, 1990). This intermediate is converted to the mature protein at the outer surface of the inner membrane. The same processing events have been observed for the sorting of $b_2$(167)-DHFR (Rassow et al., 1989; Glick et al., 1992; Koll et al., 1992). At all time points during a 40-min import reaction at the non-permissive temperature, more unprocessed precursor was detectable in the mutant mitochondria than in wild-type, and the appearance of mature $b_2$(167)-DHFR was retarded (Fig. 10, *A* and *B*). Reduced kinetics of processing were also observed for authentic cytochrome $b_2$ under these conditions (not shown). Since the processing enzyme in the matrix is fully functional in the hspl0 mutant mitochondria at the non-permissive temperature (see above), the processing defect is probably due to a reduced accessibility of the cytochrome $b_2$ presequence to the protease. Whether the altered kinetics of proteolytic maturation reflect a defect in the sorting of $b_2$(167)-DHFR to the intermembrane space remains to be seen. The accumulated precursor and intermediate-sized forms of the protein could be localized in the matrix or in the inner membrane exposed to the intermembrane space.

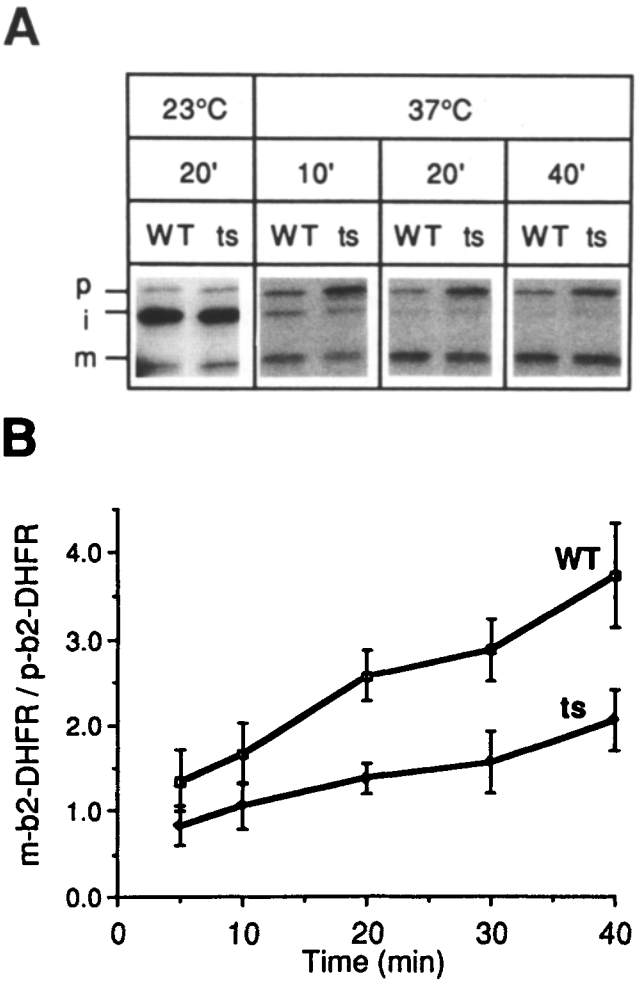



*Figure 10.* Imported $b_2$(167)-DHFR is processed with reduced kinetics in mutant mitochondria. (*A*) Processing of the radiolabeled fusion protein during import into isolated mitochondria. Aliquots of the import reactions were stopped at the times indicated by dilution into an equal volume of ice-cold BSA buffer and addition of 1 $\mu$M valinomycin. Proteinase K treatment was performed (see Fig. 5), mitochondria reisolated, and analyzed by SDS-PAGE and fluorography. (*B*) Amounts of imported protein, obtained as in *A*, were quantified with the Phosphorimager system. The ratio of imported mature (*m-$b_2$-DHFR*) to precursor form (*p-$b_2$-DHFR*) is shown over the time course of import at 37°C as the mean value of five independent experiments. Error bars indicate the standard deviation.

## *Discussion*

Our findings identify the cochaperonin Hspl0 as an essential component of the mitochondrial protein folding machinery of yeast. The interaction of Hspl0 with Hsp60 is necessary for the folding and assembly of newly imported proteins. Hspl0 also participates in maintaining certain translocated proteins, such as the Rieske Fe/S protein, in a conformation competent for further intramitochondrial sorting. The 2–3-fold inducibility of *HSP10* expression at elevated temperature is equivalent to that of *HSP60* (Cheng et al., 1989) and probably reflects the involvement of Hspl0 in the Hsp60-dependent stabilization of certain preexistent proteins under heat stress (Martin et al., 1992). During the preparation of this manuscript, the sequence of the HSP10 gene and its essential requirement for the growth of yeast was also reported by Rospert et al. (1993*b*).

The dependence of the various aspects of chaperonin function on its cochaperonin in vivo had not been analyzed previously. Using the temperature-sensitive *hspl0* mutant P36>S, we have carried out such an analysis *in organello*. A short incubation of the isolated mitochondria at 37°C was sufficient to reversibly express the mutant phenotype. Import of the matrix protease $\alpha$-MPP at the non-permissive temperature resulted in the formation of detergent-insoluble aggregates, indicating incorrect folding. Newly imported $\alpha$-MPP is known to interact with Hsp60 before its assembly with $\beta$-MPP to the functional MPP (Manning-Krieg et al., 1991). Hsp60 function is also required for the correct assembly of trimeric OTC (Cheng et al., 1989). Our data demonstrate the essential role of Hspl0 in this process in intact organelles. The primary function of the chaperonin system in these reactions is probably to mediate the folding of the imported protein subunits into a conformation competent for spontaneous oligomeric assembly. This has been demonstrated most clearly by the recent in vitro reconstitution of active OTC from the denaturant-unfolded protein achieved in the presence of GroEL and GroES (Zheng et al., 1993).

Interestingly, not all proteins imported into mitochondria show the same dependence on Hspl0 for their Hsp60-mediated folding. Cytosolic DHFR, targeted into the mitochondrial matrix as a cleavable fusion precursor, folds with normal kinetics in the *hspl0* mutant mitochondria at 37°C. A requirement of Hsp60 for DHFR folding has been demonstrated previously upon import into mitochondria in vitro and in intact cells (Ostermann et al., 1989; Martin et al., 1992). Although a residual Hspl0 activity in the P36>S mutant cannot be excluded, our finding is consistent with the observation that GroES is not required for the productive in-

teraction of unfolded DHFR with purified GroEL in vitro (Martin et al., 1991; Viitanen et al., 1991). The folding of DHFR may, however, be accelerated by Hspl0 at lower temperatures which could not be investigated in the temperature-sensitive *hspl0* mutant strain. Our results suggest, that small proteins, such as DHFR, which can refold spontaneously in vitro, may be less dependent of the cochaperonin for their Hsp60-mediated folding in vivo.

It is intriguing that the inactivation of Hspl0 also affects the biogenesis of certain proteins which pass through the matrix compartment en route to the intermembrane space. The Rieske Fe/S protein follows such a pathway (Hartl et al., 1986; Hartl and Neupert, 1990). After translocation into the matrix, the precursor is processed to an intermediate-sized form by the matrix processing enzyme, and is then cleaved to the mature size by an octapeptidyl peptidase before its export to the outer surface of the inner membrane (Isaya et al., 1992). By interacting with Hsp60, the matrix-localized intermediate is probably maintained in a loosely folded conformation competent for export (Cheng et al., 1989; Ostermann et al., 1989). Our results indicate that this function of Hsp60 is dependent on Hspl0, at least at 37°C. An "antifolding" function of Hsp60 has also been proposed to be involved in the sorting of cytochrome $b_2$ to the intermembrane space (Cheng et al., 1989; Koll et al., 1992). Such a role of Hsp60 has recently been disputed, based on the finding that proper sorting of cytochrome $b_2$ is possible in a Hsp60-deficient mutant strain (Glick et al., 1992; Hallberg et al., 1993). We find that the maturation of a cytochrome $b_2$-DHFR fusion precursor occurs with reduced kinetics in the *hspl0* mutant. While this does not indicate a requirement of Hspl0 for cytochrome $b_2$ sorting, the data would be consistent with the view that, in the presence of functional Hsp60, cytochrome $b_2$ interacts with the chaperonin system. Interestingly, a requirement for both GroEL and GroES has been reported for the export of pre-β-lactamase in *E. coli* (Kusukawa et al., 1989), which reaches the periplasmic space by a process that may be similar to the export of proteins from the mitochondrial matrix (Hartl and Neupert, 1990).

The mechanism of action of the cochaperonin has been revealed by studies of *E. coli* GroEL and GroES in vitro. Both components form an isolatable 1:1 stoichiometric complex (Langer et al., 1992*b*; Saibil et al., 1993; Todd et al., 1993), which dissociates and reassociates in ATP-dependent cycles (Martin et al., 1993*a*). Binding of GroES increases the cooperativity of ATP binding and hydrolysis by GroEL (Gray et al., 1991; Todd et al., 1993; Martin et al., 1993*b*). This is critical for the efficient release of bound substrate protein for subsequent folding, in particular for polypeptides which bind to the chaperonin with high affinity. Analysis of the temperature-sensitive *hspl0* mutant provided insight into the molecular basis of the Hspl0-Hsp60 interaction. The P36>S mutant hspl0 remains soluble and maintains the characteristic oligomeric structure at the non-permissive temperature, but has a strongly reduced ability to bind to GroEL. The mutation affects a domain of Hspl0 that is homologous to a "mobile loop" of GroES extending between residues 17–32 (residues 25–40 of Hspl0). This loop region has been proposed to represent all or part of the GroEL-binding region of the bacterial cochaperonin (Landry et al., 1993). The substitution of a glycine residue within this domain by aspartic acid (G24>D) results in a temperature-sensitive phenotype in bacteria (Landry et al., 1993) as does the homologous change in yeast Hspl0 (G32>D). Our findings stress the significance of the mobile loop domain of the cochaperonin for the functional interaction with GroEL/Hsp60. Interestingly, the P36>S mutation in Hspl0 does not result in the accumulation of substrate protein in a complex with Hsp60, indicating that protein release from the chaperonin is possible in the absence of Hspl0 function. However, this release is not productive for folding and leads to aggregation, except for small proteins such as DHFR. These data are consistent with the function of Hspl0 in synchronizing the ATP-dependent release activity of the individual chaperonin subunits.

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