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 hsplO 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 hspl0 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., 1992a), 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 (Ssclp 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 (Osterman 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, 1993a). 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., 1992b; 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., 1993a, 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 (Lamenet 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., 1993a). 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-
chymotryptic digestion was performed, the resulting peptides were S-alkyl-
genomic fragments of the identified plasmids was determined by means of
YCp50-based genomic library of
Jagow (1987). After electroblotting onto nitrocellulose membrane, the Pon-
tem. Fractions were analyzed by SDS-PAGE according to Soh/igger and yon
2 % peptone, 3 % glycerol). Mitochondria were isolated as described (Daum
mM ATP, 10 mM MgCl2), respectively. Superose 6 gel filtration chroma-
cated with 4-vinylpyridine, and separated by narrow bore (2.1 ram) reversed
carry out using an optimized 477A instrtlment (Appl. Biosystems, Inc.,
sham Corp., Arlington Heights, IL) and subsequently used to screen a
HSPIO, (MATa, leu2-3, trpl-1, ura3-52).
Expression of HsplO-/~-galactosidase was analyzed in
HSPIO,
canl-lO0, his3-11, his3-15, leu2-3, leu2-112, trpl-1, ura3-5, AhsplO: :LEU2,
HSPIO
cloning and Sequencing of HSPIO
HSPIO,
canl-lO0, his3-11, his3-15, leu2-3, leu2-112, trpl-1, ura3-5, AhsplO: :LEU2,
HSPIO/HSPIO
Expression of Hspl0-/~galactosidase was analyzed in
strain UTL-7A (MATa, leu2-3, trpl-1, ura3-52).

Purification of Hspl0
D273-10B cells were grown overnight in YPG medium (1% yeast extract,
2 % peptone, 3 % glycerol). Mitochondria were isolated as described (Daum
mM Tris-HC1 (pH 7.3), 1 mM EDTA, 10 mM KC1 (buffer A) and lysed by sonicati-
ond bacterial clones were obtained and their plasmid DNA prepared. The
m-ScaI fragraent into the

Cloning and Sequencing of HsplO
To amplify HsplO, a PCR reaction was performed according to Innis et al.
(1990). Yeast genomic DNA of wild-type strain D273-10B, isolated as de-
scribed (Ausubel et al., 1989), was used as template. The amplified HsplO
fragment was labeled using the Megaprime DNA labeling system (Amers-
short Corp., Arlington Heights, IL) and subsequently used to screen a
Ycp50-based genetic library of Saccharomyces cerevisiae (Rose et al.,
1987; Ausubel et al., 1989). The localization of the HsplO gene within the
genomic fragments of the identified plasmids was determined by means of
Southern hybridization after restriction digest (Ausubel et al., 1989). The
HsplO locus surrounding an internal SmaI-restriction site was sequenced
with Sequenase (United States Biological 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 HsplO-lacZ Fusion and Analysis of Expression
A 284-bp HindIII-Smal fragment comprising the 5' non-coding region and
classes of the open reading frame of HsplO was subcloned into YEp356R
(Myers et al., 1986). The resulting construct encodes a fusion protein con-
taining amino acids 1-53 of HsplO and full length ~-galactosidase under
control of the HsplO 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
Aubel et al. (1989)), and then diluted to an OD600 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
~galactosidase activity measured (Ausubel et al., 1989).

Gene Replacement
A one-step gene replacement was carried out as published (Rothstein,
1983). In brief, a ~380-bp HindIII-Scl fragment containing HsplO was
subcloned into pBluescript SK+ (Stratagene). This construct was used as
a template in two independent PCR reactions to amplify regions surround-
HsplO. 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-
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
the protocol of Gietz and Sugino (1988). Integration into the HsplO gene locus was confirmed by Southern hybridization.
A HsplO/hsplO diploid was then sporulated and tetrads analyzed for spore
viability. The diploid strain was also transformed with the 380-bp Hind-
III-Scl fragment containing HsplO in the URA3 marker vector pRS316
(WT-HsplO). After sporulation and tetrade dissection Hspl0/hsplO haploid s
be isolated carrying a plasmid-derived HsplO gene (Hspl0/WT-HsplO).

Screening for a Temperature-Sensitive hsplO Mutant
A plasmid shuffling experiment (Ausubel et al., 1989) was performed to
identify temperature-sensitive hsplO mutants after chemical mutagenesis of
the isolated HsplO. Hydroxylamine mutagenesis of the plasmid WT-HsplO
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 380-bp Hind-
III-Scl fragment into the HIS3-containing vector pRS413. 3,500 indepen-
dent bacterial clones were obtained and their plasmid DNA prepared. The
haploid yeast strain Hspl0/WT-HsplO was transformed with this muta-
gized plasmid library. Yeast transformants were replica-plated onto medium
containing 5-fluoroorotic acid and uracil to induce a loss of the plasmid WT-
HsplO and incubated at 23°C (according to Ausubel et al., 1989). The
resulting haploid s containing exclusively the mutagenized form of the
HsplO 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 HsplO 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
HsplO strains transformed with the wild-type HsplO gene (WT-HsplO) and
the temperature sensitive allele (ts-hsplO), respectively, were grown over-
night at 23°C in YPGal medium (1 % yeast extract, 2 % peptone, 2 % galac-
tose) to an OD600 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 concentra-
tion 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 [35S]methionine in reticulocyte lysate (Promega). After translation,
postribosomal supernatants were prepared according to Zimmermann and

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Neupert (1980). Import reactions contained 10–20% postribosomal supernatants, 0.4 mg/ml mitochondria, 2 mM ATP, 2 mM NADH, and 4 mM MgCl₂, 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)-Sepharose (Hoheneraad 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 MgCl₂, in BSA-buffer (3% BSA, 220 mM sucrose, 80 mM KC1, and 10 mM MOPS, pH 7.2). Inhibition of the membrane potential and protease activity was analyzed by measuring the membrane potential and protease activity using a DPH fluorescence polarization assay (Koll et al., 1992).

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. Size-fractionated soluble mitochondrial proteins of 10–300 kD 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.

In Vitro Transcription/Translation of HSPIO

A BamHI restriction site was introduced upstream of the HSPIO as a template. The obtained PCR product was sequenced to confirm its wild-type character and a BamHI–Sal fragment containing HSPIO subcloned into pGEM (Promega). In a similar way the ts-allele was amplified using the plasmid ts-hsplO 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 pOPF39 (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 MgCl₂ and 0.2 mM ATP.

Cloning and Sequencing of the Yeast HSPIO 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 HSPIO 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 HSPIO 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 nitrocellulose membrane and subjected to trypptic 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 (Vitanen et al., 1990; Langer et al., 1992b; Hartman et al., 1992).

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.

Figure 2. A: Identification of the yeast mitochondrial Hsp10 gene. 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.
liver Hsp10. A sequence alignment of the three proteins is presented in Fig. 2 B.

The NH2-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 α-helical conformation for this region. These are the hallmark features of mitochondrial targeting sequences that direct proteins into the matrix (Hartl et al., 1989). However, this 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 *HSPI0* 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 *HSPI0* 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 *HSPI0* is an essential gene at normal growth temperature. The *HSPI0Δhspl0* diploid was also transformed with a wild-type copy of the *HSPI0* gene on a *URA3* containing plasmid before sporulation. *LEU2/URA3* haploids were isolated which were unable to grow at 23°C or 37°C. Upon shift to the elevated temperature, a 2.3-fold induction of β-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 55°C (data not shown). These results suggest that the transcription of *HSPI0* 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.

**Induction of HSPI0 Expression upon Temperature Shift**

The 5′ non-coding region of the *HSPI0* 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 *HSPI0* gene (Fig. 2A). However, they are separated by 5-10 nucleotides. To test whether Hsp10 expression is heat inducible, an *HSPI0-lacZ* fusion was constructed which encodes the amino terminal 53 residues of Hsp10 followed by β-galactosidase under control of the *HSPI0* 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 3.2-fold induction of β-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 55°C (data not shown). These results suggest that the transcription of *HSPI0* 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 2.** (A) Nucleotide sequence of the *HSPI0* 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 results suggest that the transcription of *HSPI0* 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.
Isolation of Temperature-sensitive Alleles of Hspl0

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-HSPIO, the HSPIO gene was subcloned into a HIS3 single copy vector. The resulting library of mutagenized hspl0 was subsequently transformed into the haploid Δhspl0 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. 4A). The ts-phenotype was not observed when these clones were incubated at both temperatures before counterselection against the wild-type copy of the HSPIO 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. 4B). The proline residue at this position is highly conserved in several GroES homologues with the exception 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).

Mutant hspl0 Has a Reduced Binding Affinity for the Chaperonin

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. 4B). 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. 4B). When the G32>D mutation was introduced into HSPIO, 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 cochaperonin.
presence of MgATP resulted in the highly efficient formation of the interaction indicates that the cochaperonin was bound to GroEL was added to the mitochondrial lysates and the formation of the Hspl0-GroEL complex was analyzed by native PAGE. When the extract was depleted of ATP by apyrase treatment, no interaction between Hspl0 and GroEL was detectable at the elevated temperature (not shown). These data demonstrate that P36>S hsp10 has a reduced affinity for the chaperonin under non-permissive conditions. This decrease in hsp10 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 Hsp10 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 hsp10.

Requirement of Hspl0 for Folding of Imported Proteins

The temperature-sensitive yeast strain carrying the P36>S mutation in hsp10 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 Δhsp10 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 precubation 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 oligomer were prepared and the ability of Hsp10 to associate with the chaperonin was analyzed. Based on the observation that the interaction of yeast mitochondrial Hsp10 with GroEL is fully functional (see above; Rospert et al., 1993a), 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 Hsp10 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., 1992b). 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 Hsp10 and endogenous Hsp60 was observed (not shown). However, this interaction was less efficient than binding of Hsp10 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 hsp10 that bound to GroEL at 37°C was diminished by 80%. Size exclusion chromatography at 37°C demonstrated that the mutant hsp10 oligomer does not dissociate at the elevated temperature (not shown). These data demonstrate that P36>S hsp10 has a reduced affinity for the chaperonin under non-permissive conditions. This decrease in hsp10 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.

Figure 5. P36>S hsp10 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 (WTts), and mutant hsp10 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 KCI to a protein concentration of 0.25 mg/ml, and divided into aliquots containing equal amounts of imported Hsp10. 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 MgCl₂ (+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 Hsp10 and GroEL was observed. The complex migrates with a reduced mobility relative to GroEL alone.
In the P36>S strain (ts) mitochondria at 37°C for 25 min. Mitochondria were isolated 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.

Figure 7. Inactivation of Hsp10 affects the assembly of rat OTC in isolated yeast mitochondria. Radiolabeled precursor of rat ornithine transcarbamylase was imported into wild-type (WT) and mutant (ts) mitochondria at 37°C for 25 min. Mitochondria were pretreated at 37°C for 30 min. After import, further incubation of the inactivated mutant mitochondria at 37°C, no aggregation of o-MPP was observed (Fig. 6 C). This is consistent with the finding that the reduction in binding of P36>S hsp10 to the chaperonin at 37°C is fully reversible (see above). It cannot be excluded, however, that the folding of α-MPP requires Hsp10 function only at the elevated temperature.

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 hsp10 mutant organelles under non-permissive conditions (Fig. 7), demonstrating the essential role of Hsp10 for the assembly of OTC.
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 F0-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 cochaperonin 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 b2 followed by the complete sequence of mouse dihydrofolate reductase (b2(167)-DHFR) was also found to be affected in the P36>S
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 hsp10 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.

**Discussion**

Our findings identify the cochaperonin Hsp10 as an essential component of the mitochondrial protein folding machinery of yeast. The interaction of Hsp10 with Hsp60 is necessary for the folding and assembly of newly imported proteins. Hsp10 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 Hsp10 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).

The dependence of the various aspects of chaperonin function on its cochaperonin in vivo had not been analyzed previously. Using the temperature-sensitive hsp10 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 Hsp10 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 Hsp10 for their Hsp60-mediated folding. Cytosolic DHFR, targeted into the mitochondrial matrix as a cleavable fusion precursor, folds with normal kinetics in the hsp10 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 Hsp10 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-

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**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 mM 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.
teration of unfolded DHFR with purified GroEL in vitro (Martin et al., 1991; Vitanen et al., 1991). The folding of DHFR may, however, be accelerated by Hsp10 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 Hsp10 also affects the biogenesis of certain proteins which pass through the matrix compartmen 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 Hsp10, at least at 37°C. An "antifolding" function of Hsp60 has also been proposed to be involved in the sorting of cytochrome b1 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 b1 is possible in a Hsp60-deficient mutant strain (Glick et al., 1992; Hallberg et al., 1993). We find that the maturation of a cytochrome b1-DHFR fusion precursor occurs with reduced kinetics in the hsp10 mutant. While this does not indicate a requirement of Hsp10 for the cytochrome b1 sorting, the data would be consistent with the view that, in the presence of functional Hsp60, cytochrome b1 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., 1992b; Saibil et al., 1993; Todd et al., 1993), which dissociates and reassociates in ATP-dependent cycles (Martin et al., 1993a). Binding of GroES increases the cooperativity of ATP binding and hydrolysis by GroEL (Gray et al., 1991; Todd et al., 1993; Martin et al., 1993b). 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 Hsp10-Hsp60 interaction. The P36>S mutant hsp10 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 Hsp10 that is homologous to a "mobile loop" of GroES extending between residues 17-32 (residues 25-40 of Hsp10). 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 Hsp10 (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 Hsp10 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 Hsp10 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 Hsp10 in synchronizing the ATP-dependent release activity of the individual chaperonin subunits.

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