The chaperonin-related protein Tcm62p ensures mitochondrial gene expresssion under heat stress

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Abstract Tcm62p, distantly related to chaperonins, is required for the assembly of succinate dehydrogenase in mitochondria of *Saccharomyces cerevisiae* and was proposed to exert chaperone activity. We demonstrate here crucial functions of Tcm62p under heat stress. It ensures mitochondrial gene expression at elevated temperatures and prevents heat-aggregation of the ribosomal subunit Var1p. Similar to chaperonins, Tcm62p forms a high molecular mass protein complex of approximately 850 kDa in the mitochondrial matrix space. These results suggest a more general chaperone function of Tcm62p in mitochondria.

Key words: Tcm62p; Succinate dehydrogenase; Chaperonin; Mitochondrion; Heat stress

1. Introduction

Chaperonins play fundamental roles in mediating protein folding in all organisms [1–4]. Similar to other molecular chaperone proteins, they bind non-native protein structures and stabilize otherwise unstable conformers against aggregation. Chaperonins are high molecular mass protein complexes composed of subunits of approximately 60 kDa [5–7]. They form double-ring assemblies which allow ATP-dependent protein folding to occur in a sequestered environment. Two structurally related classes can be distinguished [1]: Members of the Hsp60/Cpn60 family, *Escherichia coli* GroEL being the paradigm, are ubiquitous in eubacteria and in endosymbiontic organelles of eukaryotic cells. A distinct class of chaperonins, the thermosome/TRiC/CCT-family, occurs in archaebacteria and the eukaryotic cytosol. Although similar in oligomeric structure, they display only limited similarity in primary structure to Hsp60/Cpn60 proteins [8,9].

Studies on the biogenesis of mitochondria revealed early insights into the role of chaperonins for protein folding in vivo. Hsp60, localized in the matrix space, mediates folding of many nuclear-encoded proteins after their import into mitochondria [10–12]. Similarly, folding of mitochondrially encoded proteins is facilitated by Hsp60 [13]. Under heat stress conditions, the chaperonin is required to prevent the aggregation of thermolabile proteins and maintain them in an active conformation [14].

While Hsp60 assists folding of many mitochondrial proteins, a number of other helper proteins with apparently more specific functions have been identified in mitochondria of *Saccharomyces cerevisiae* which ensure the posttranslational assembly of respiratory chain complexes without being themselves part of these complexes [15,16]. These factors include enzymes involved in the synthesis of prosthetic groups, proteins mediating membrane insertion of respiratory chain subunits and potential chaperone proteins which facilitate complex assembly.

Tcm62p (for *t*ric*a*rboxylic acid cycle *m*utant) has been identified in a genetic screen for mutants affecting the biogenesis of succinate dehydrogenase (SDH) [17]. Cells lacking Tcm62p are respiratory deficient and lose SDH oxidase activity. The *TCM62* gene encodes a protein of 64.7 kDa which is distantly related to chaperonins. It shares 18% sequence identity and 38% sequence similarity with the mitochondrial chaperonin Hsp60 of *S. cerevisiae* which is the only related protein in this organism. Taking this sequence similarity into account, a chaperone function of Tcm62p during the assembly of yeast SDH has been proposed [17].

Here, we have further characterized Tcm62p in mitochondria. We demonstrate that Tcm62p is part of a large protein complex in the mitochondrial matrix with a molecular mass similar to that of chaperonins. Tcm62p is crucial for mitochondrial functions at high temperature. Deletion of *TCM62* impairs respiratory competence and mitochondrial translation under these conditions and leads to the heat-aggregation of the ribosomal subunit Var1p. We therefore propose that Tcm62p represents a novel molecular chaperone which is required for the thermoprotection of mitochondria.

2. Materials and methods

2.1. Yeast strains and growth conditions

Yeast strains used in this study were derivatives of W303 if not indicated otherwise. *TCM62* was disrupted in the diploid strain FY1679 by PCR-targeted homologous recombination using the heterologous marker *KanMX4* [18]. The complete *TCM62* gene was replaced by the disruption cassette. Homologous recombination was verified by PCR. Haploid Δ*tcm62* segregants were isolated by sporulation and tetrad dissection. In parallel, *TCM62* was disrupted in the haploid strain W303-1A. The derived Δ*tcm62* strain YCK1 (*MAT**a** ade2-1 his3-11,15 trp1-1 leu2,112 ura3-52 crc1::KanMX4*) was used routinely in the experiments described here.

For preparation of mitochondria, cells were grown at 24°C on lactate medium. For labelling of mitochondrial translation products in vivo, cells were grown on methionine-free minimal medium (0.7% yeast nitrogen base containing ammonium sulfate) containing 3% glycerol and supplemented with the auxotrophic requirements.

2.2. Cloning of TCM62

TCM62 was amplified by PCR and cloned into the vector pCR™2.1 (Invitrogen) utilizing single adenosine overhangs of the PCR product. A 1.7 kb fragment containing *TCM62* was isolated by restriction digestion of pCR™2.1-*TCM62* with *Bam*HI and *Sal*I

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Abbreviations: SDH, succinate dehydrogenase

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and cloned into pGEM4 (Promega) to be used for in vitro synthesis of the protein (pGEM4-*TCM62*). The sequence of *TCM62* differed from that deposited in the database after position 1698. An additional adenosine nucleotide is present at this position in *TCM62*. This observation was verified by cloning the genomic fragment of *TCM62* from W303 by gap repair. The frame shift caused by the additional nucleotide results in the alteration of the C-terminus of Tcm62p: The C-terminal eight amino acid residues are replaced by the heptameric peptide KPERHKA.

2.3. Expression of TCM62 in E. coli

For generation of a polyclonal antiserum against Tcm62p, the protein was expressed in *E. coli*. pGEM4-*TCM62* was digested with *Bam*HI and *Hin*dIII. The resulting 1.7 kb DNA-fragment containing the *TCM62* gene was cloned into pQE9 (Quiagen). After expression in the *E. coli* strain XL1blue, inclusion bodies of Tcm62p were isolated according to standard procedures and used for generation of antibodies in rabbits.

2.4. Subfractionation of mitochondria

Mitochondria were isolated according to published protocols [19,20]. To disrupt the mitochondrial outer membrane, mitochondria were reisolated by centrifugation, resuspended in 20 mM HEPES/KOH pH 7.4, 50 mM potassium acetate at a concentration of 50 μg protein/ml and incubated for 30 min at 4°C in presence or absence of proteinase K (30 μg/ml). Protease digestion was halted by adding PMSF to a final concentration of 1 mM followed by an incubation of the samples for 3 min at 4°C. For alkaline extraction, mitochondria were resuspended in 0.1 M Na_2CO_3 at a concentration of 75 μg/ml. After an incubation for 30 min at 4°C, the sample was centrifuged for 60 min at 100 000 × *g*. Soluble and pellet fractions were analyzed by SDS–PAGE and immunoblotting using a chemiluminescence detection system.

3. Results

3.1. Tcm62p is localized in the mitochondrial matrix space

To allow a further characterization of Tcm62p, a polyclonal antiserum directed towards this protein was raised. This antibody recognized a single band of approximately 60 kDa in wild type mitochondria which was not detected in mitochondria isolated from a *tcm62*-null cells (data not shown). The analysis of mitochondrial subfractions using this antiserum revealed a localization of Tcm62p in the matrix space. The protein remained completely resistant towards added proteinase K upon osmotic disruption of the mitochondrial outer membrane (Fig. 1). Tcm62p shared this behavior with various mitochondrial matrix proteins, whereas the soluble intermembrane space protein cytochrome *c* peroxidase was released into the supernatant fraction under these conditions (Fig. 1). Lysis of the mitochondrial inner membrane by adding detergent prior to proteinase K, however, resulted in complete degradation of Tcm62p (data not shown). Hydrophobic segments, which might serve as membrane anchors, are not present in the sequence of Tcm62p indicating that it is not an integral membrane protein. Consistently, Tcm62p was recovered in the supernatant fraction after extraction of mitochondrial membranes at alkaline pH (Fig. 1). These results demonstrate that Tcm62p is localized in the mitochondrial matrix space. In contrast to our findings, Tcm62p has previously been proposed to represent an integral inner membrane protein which exposes 81 C-terminal amino acid residues to the intermembrane space [17]. These studies have been performed in mitochondria isolated from *tcm62*-null cells overexpressing a Tcm62p-derivative with a C-terminal hemagglutinin-tag. While our results do not exclude a peripheral association of Tcm62p with the inner surface of the mitochondrial inner membrane, they make the presence of a transmembrane region in Tcm62p highly unlikely. A proteolytic fragment lacking C-terminal parts of Tcm62p did not accumulate upon protease treatment of mitoplasts (Fig. 1). Moreover, radiolabelled Tcm62p, which was posttranslationally imported into isolated mitochondria, showed a similar behavior as endogenous Tcm62p upon subfractionation of mitochondria and remained completely protected against protease in mitoplasts (Fig. 1).

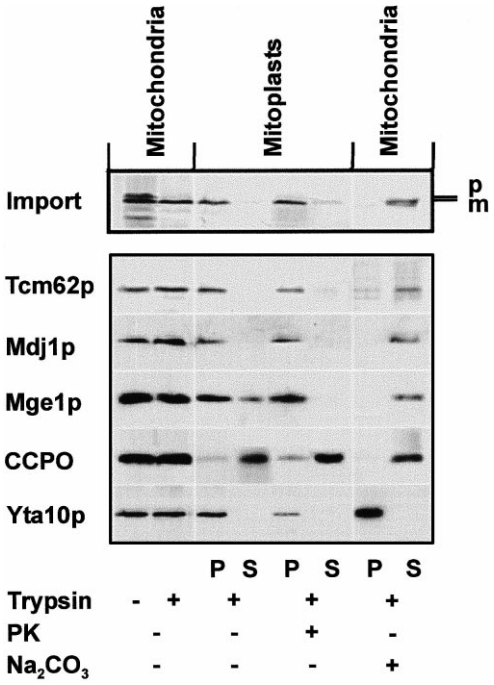

Fig. 1. Tcm62p is a mitochondrial matrix protein. Tcm62p was synthesized in reticulocyte lysate in the presence of ^{35}S-methionine according to standard procedures and imported into isolated mitochondria for 30 min at 25°C as described [29]. Non-imported preproteins were digested with trypsin (100 μg/ml) for 15 min at 4°C. The protease was inhibited by adding soybean trypsin inhibitor (1 mg/ml). Subsequently, mitochondria were subfractionated. Mitoplasts were generated and membrane association of Tcm62p was examined by alkaline extraction ('Na_2CO_3'). The various fractions were analyzed by immunoblotting with antibodies directed against Tcm62p, the matrix proteins Mdj1p and Mge1p, the intermembrane space protein cytochrome *c* peroxidase ('CCPO') and the integral inner membrane protein Yta10p. The upper box shows the autoradiograph of the gel. p, precursor form of Tcm62p; m, mature form of Tcm62p.

3.2. Tcm62p is part of a large complex in mitochondria

Chaperonins form homo- or hetero-oligomeric high molecular mass protein complexes [1]. The sequence similarity of Tcm62p to chaperonins therefore prompted us to determine the native molecular mass of Tcm62p in mitochondria. Isolated mitochondria were solubilized and extracts were subjected to gel filtration analysis. The eluate fractions were analyzed by immunoblotting using a Tcm62p-specific antiserum. Tcm62p eluted from the column in a single peak which corresponded to a molecular mass of approximately 850 kDa (Fig. 2). Thus, Tcm62p is part of a complex in mitochondria which has a molecular mass similar to that of chaperonins. The mitochondrial chaperonin Hsp60 coeluted with Tcm62p from the column as revealed by Western blot analysis (Fig. 2). This observation raises the possibility that Tcm62p and Hsp60 are part of the same complex. Tcm62p, however, was not

detectable in the immunoprecipitate of mitochondrial extracts using Hsp60-specific antibodies, nor was Hsp60 coimmunoprecipitated with Tcm62p-specific antibodies (data not shown). These results suggest that Tcm62p and Hsp60 form two independent high molecular mass protein complexes of similar size in mitochondria.

A complex of Tcm62p with SDH-subunits has been identified in cells overexpressing a HA-tagged derivative of Tcm62p [17]. We therefore analyzed eluate fractions of the gel filtration column with a polyclonal antiserum directed against subunit 2 of SDH (Sdh2p). Sdh2p did not cofractionate with Tcm62p and eluted in a single peak in fractions corresponding to a molecular mass of approximately 150 kDa (Fig. 2). We conclude that Tcm62p is part of a large complex in wild type cells which does not contain Sdh2p.

3.3. *Tcm62p is required for respiratory competence at high temperature*

For further characterization of the role of Tcm62p in mitochondria, the growth phenotype of a *tcm62*-null mutant strain was examined. In agreement with previous findings [17], cells lacking Tcm62p did not exhibit a growth defect on fermentable carbon sources at any temperature tested (data not shown). We noted, however, a striking temperature-sensitive growth of *tcm62*-null cells on rich glycerol medium: while growth of Δ*tcm62* cells was significantly retarded at 24°C relative to wild type cells, it ceased completely at 37°C (Fig. 3). These results demonstrate that Tcm62p is essential for the maintenance of the mitochondrial respiratory function at higher temperatures and thereby point to crucial functions of Tcm62p under heat stress.

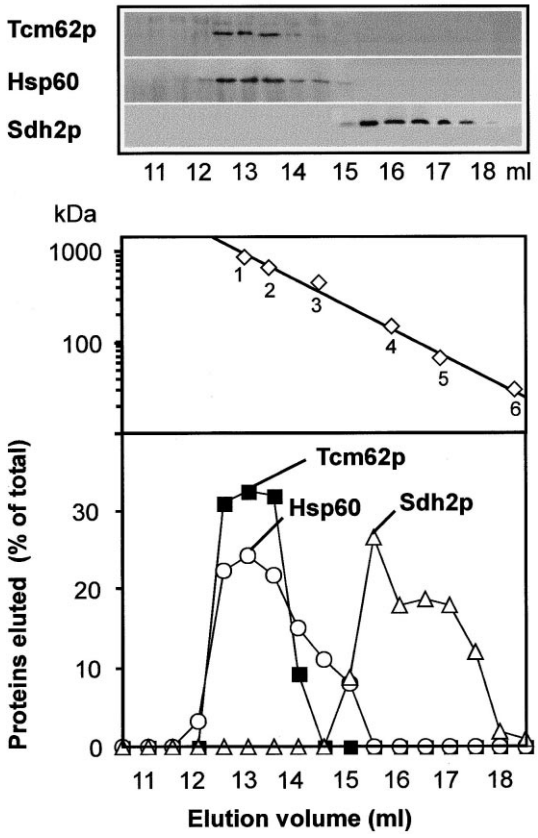

Fig. 2. Tcm62p is part of a large protein complex in mitochondria. Mitochondria (600 μg) were resuspended in 0.1% Triton X-100, 30 mM HEPES/KOH pH7.4, 150 mM potassium acetate, 1 mM PMSF at a concentration of 2 mg protein/ml and lysed by vigorous shaking for 15 min at 4°C. After a clarifying spin for 15 min at 100 000 × *g*, the supernatant fraction was loaded onto a Superose 6 column equilibrated with lysis buffer. Fractions of 0.5 ml were collected, TCA-precipitated and analyzed by SDS–PAGE and immunoblotting with Tcm62p-, Hsp60- and Sdh2p-specific antisera. The immunoblot is shown in the upper panel. The amount of Tcm62p, Hsp60 and Sdh2p in various fractions was quantified by laser densitometry and is given as percent of total eluate in the lower panel. Hsp60 (1), thyroglobulin (2), apoferritin (3), alcohol dehydrogenase (4), bovine serum albumin (5) and carboanhydrase (6) were used for calibration.

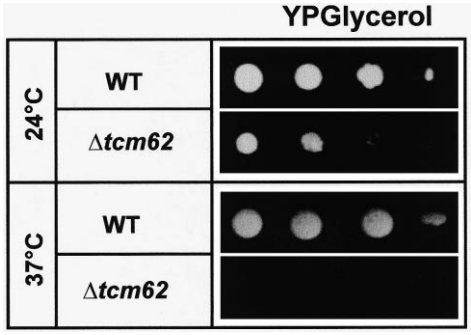

Fig. 3. Tcm62p is required for mitochondrial respiratory function at 37°C. Wild type (WT) and *tcm62*-null cells were grown on YP medium containing 3% glycerol at 24°C to mid-log phase. 10-fold serial dilutions of cells were spotted onto YP-plates containing 3% glycerol (YPGlycerol) and incubated at 24 or 37°C for 4 days.

3.4. *The thermostability of mitochondrial protein synthesis depends on Tcm62p*

Previous studies have revealed that the synthesis of mitochondrially encoded proteins is a thermosensitive process which is protected against thermal inactivation by heat shock proteins [21]. We therefore examined the thermostability of mitochondrial protein synthesis in cells lacking Tcm62p. Wild type and *tcm62*-null cells were grown under respiring conditions at 24°C to mid-log phase. Cells were then shifted to various temperatures and mitochondrially encoded proteins were selectively labelled with ^{35}S-methionine. In wild type cells, the efficiency of mitochondrial protein synthesis increased approximately 1.5-fold at growth temperatures up to 37°C but strongly decreased at temperatures above 40°C (Fig. 4 and data not shown). Stabilization of mitochondrial protein synthesis at temperatures up to 37°C is most likely caused by heat shock proteins which accumulate at increased levels in cells under these conditions. Interestingly, we observed a strongly increased thermosensitivity of the mitochondrial protein synthesis apparatus in the absence of Tcm62p. In contrast to wild type cells, labelling of mitochondrially encoded proteins in *tcm62*-null cells decreased at 30°C and ceased almost completely at 37°C (Fig. 4). This effect was not caused by the impaired respiratory competence of these cells at 37°C as we did not observe any effect on the thermostability of protein synthesis in Δ*yme1* cells (data not shown) which exhibit a temperature sensitive *petite* phenotype similar to Δ*tcm62* cells [22]. These results assign a crucial role to Tcm62p in stabilizing mitochondrial protein synthesis against thermal inactivation. An impaired synthesis of mitochondrially encoded proteins provides an explanation for the observed respiratory deficiency of *tcm62*-null cells at high temperature as subunits

of respiratory chain complexes and of the F_1F_O-ATPase are encoded in the mitochondrial genome [15,23].

3.5. *Tcm62p prevents the aggregation of the ribosomal subunit Var1p at high temperature*

In view of its sequence similarity to Hsp60 and other chaperonins, it is conceivable that Tcm62p exerts chaperone activity in mitochondria and thereby ensures the thermoprotection of mitochondrial protein synthesis. To obtain further insights into the function of Tcm62p, we analyzed the solubility of the ribosomal subunit Var1p at high temperatures. In *S. cerevisiae*, the ribosomal subunit Var1p represents the only soluble protein encoded by the mitochondrial genome [15,23]. Mitochondria were isolated from wild type and *tcm62*-null cells and mitochondrial translation products were labelled with ^{35}S-methionine at 24°C. After a subsequent incubation at 24 or 37°C, mitochondria were lysed and extracts were centrifuged to separate soluble from aggregated Var1p. When mitochondria were incubated at 24°C after labelling, Var1p was almost exclusively recovered from the supernatant fraction irrespective of the presence of Tcm62p in mitochondria (Fig. 5). Var1p remained also soluble in wild type mitochondria at 37°C (Fig. 5). In contrast, Var1p accumulated in the pellet fraction of *tcm62*-null mitochondria under these conditions indicating aggregation (Fig. 5). We conclude that Tcm62p is required to maintain the ribosomal protein Var1p in a soluble state pointing to a chaperone activity of Tcm62p.

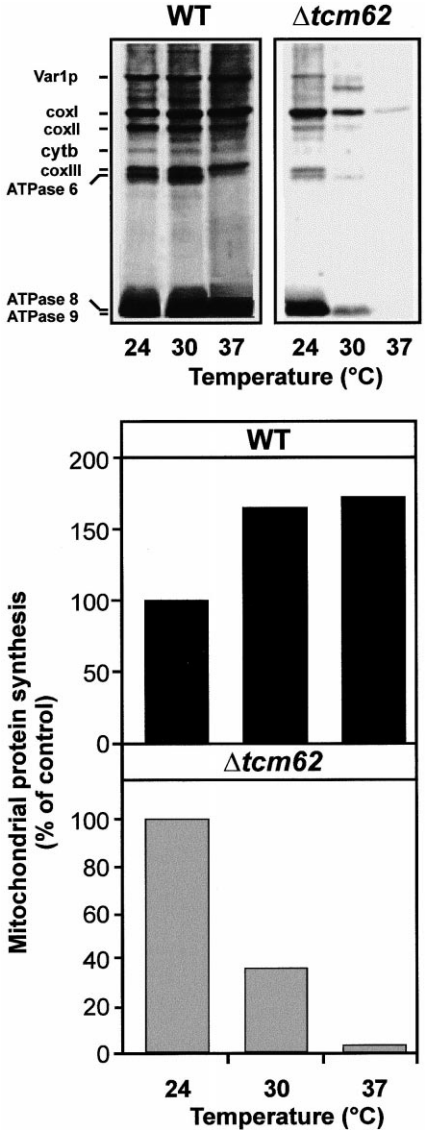

Fig. 4. Decreased thermostability of mitochondrial protein synthesis in the absence of Tcm62p. Wild type (WT) and *tcm62*-null cells were grown to mid-log phase at 24°C on synthetic medium lacking methionine and containing glycerol (3%) as the sole carbon source. Cultures were then shifted to the temperature indicated and cells were incubated for 2 h. After addition of cycloheximide (150 μg/ml), the cultures were supplemented with ^{35}S-methionine (80 μCi/ml) and mitochondrial translation products were labelled for 15 min at the indicated temperatures. Translation was inhibited with chloramphenicol (500 μg/ml) and cold methionine (4 mM) was added. After alkaline lysis of the cells, extracts were subjected to SDS–PAGE and labelling of mitochondrial proteins was assessed by fluorography (upper panel). Incorporation of ^{35}S-methionine was quantified using a phosphorimaging system and is given as percent of labelling at 24°C in the lower panel.

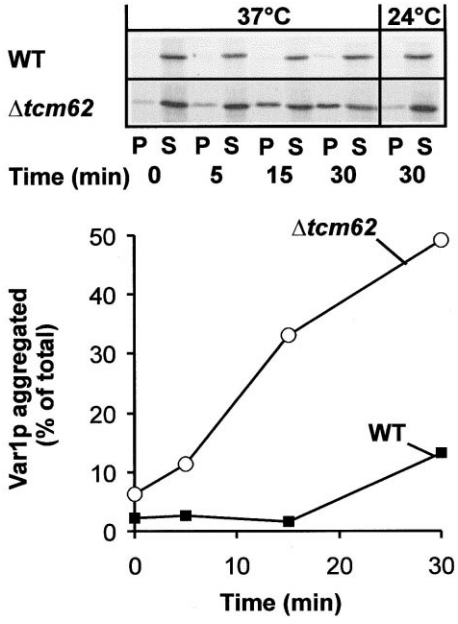

Fig. 5. Aggregation of Var1p in Δ*tcm62* mitochondria at 37°C. Mitochondrial translation products were labelled in mitochondria isolated from wild type (WT) or Δ*tcm62* cells according to published procedures [30]. Incorporation of ^{35}S-methionine was prevented by adding cold methionine (40 mM). Mitochondria were then incubated either at 24°C for 30 min or at 37°C for the time points indicated. To assess the solubility of newly synthesized Var1p [31], mitochondria (0.25 mg/ml) were lysed in 0.1% Triton X-100, 10 mM Tris–HCl pH7.4, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF. After an incubation for 10 min at 4°C, extracts were splitted into a soluble and pellet fraction by centrifugation for 10 min at $30\,000 \times g$. Both fractions were subjected to SDS–PAGE and analyzed by fluorography. The upper panel shows the fluorogram of Var1p in wild type and Δ*tcm62* mitochondria. The amount of aggregated Var1p was determined using a phosphorimaging system and is given as percent of total Var1p in the soluble and pellet fractions in the lower panel.

4. Discussion

The chaperonin-related protein Tcm62p fulfills crucial functions in mitochondrial biogenesis and is required for the assembly of the SDH-complex in the inner membrane. We describe here an additional role of Tcm62p for the protection of

mitochondrial functions under heat stress. Our results reveal structural and functional similarities of Tcm62p to chaperonins and thereby substantiate the significance of the low sequence similarity to this class of molecular chaperones.

Several lines of evidence suggest that Tcm62p might be functionally related to chaperonins: First, Tcm62p is part of a protein complex of approximately 850 kDa. A similar native molecular mass has been determined for all chaperonins which provide a sequestered cavity for folding polypeptides [1–4]. The Tcm62p-containing complex in the matrix of mitochondria apparently does not contain Hsp60-subunits as we did not detect any interaction of Tcm62p with Hsp60 in coimmunoprecipitation experiments. Second, similarities between Tcm62p and chaperonins are not restricted to the primary structure but appear to extend also to the secondary structure. Three domains can be distinguished in chaperonins of the Hsp60/Cpn60 family [5,6]: The apical domain mediating binding of polypeptides and of Hsp10/Cpn10 co-chaperonins, the equatorial domain containing the ATP-binding site and an intermediate domain which connects the two other domains. Secondary structure predictions using the program PREDATOR and taking into account the known crystal structure of *E. coli* GroEL [6] revealed significant similarities between Tcm62p and chaperonins in the N-terminal region which corresponds to parts of the intermediate and apical domains in GroEL (unpublished observations). It is therefore conceivable that, despite the sequence divergence, Tcm62p and chaperonins exhibit a similar fold in this region. Notably, the corresponding region in *E. coli* GroEL includes helix H which is directly involved in substrate binding to the chaperonin [24]. Third, the maintenance of mitochondrial respiratory function is strictly dependent on Tcm62p at high temperature, i.e. conditions which result in thermal destabilization of mitochondrial proteins. In the absence of Tcm62p, we observed an increased thermosensitivity of mitochondrial protein synthesis which has previously been reported to be stabilized against thermal inactivation by heat shock proteins [21]. Interestingly, the ribosomal protein Var1p was prone to heat aggregation in Δ*tcm62* mitochondria suggesting that Tcm62p, or other unidentified subunits of the Tcm62p-containing complex, might directly stabilize heat inactivated polypeptides.

Various chaperone proteins have been identified which exert a protective function in the mitochondrial matrix under heat stress. This includes Hsp60, which prevents protein denaturation at high temperatures [14], the mitochondrial DnaJ homologue Mdj1p [25] and the ClpB-homologue Hsp78 [26], which exerts chaperone activity and confers thermotolerance to mitochondria [21,27]. Notably, both Hsp78 and Tcm62p affect mitochondrial protein synthesis under heat stress. However, they appear to act at different stages. In contrast to Tcm62p, Hsp78 is not required for the thermostability of mitochondrial protein synthesis, but plays an essential role in its reactivation after heat stress [21]. Moreover, mitochondrial thermotolerance, while depending on Hsp78, was not impaired in cells lacking Tcm62p (data not shown).

It should be noted that amino acid residues of chaperonins involved in ATP-binding and hydrolysis are not conserved in Tcm62p suggesting an ATP-independent mode of action. In view of the modular domain structure of chaperonins, it is conceivable that only the activity of single domains of chaperonins is conserved in Tcm62p which could exert related functions in different cellular processes. Indeed, the apical domain of GroEL has been demonstrated to exhibit chaperone activity regardless of the formation of a GroEL-oligomer [28]. Studies of Tcm62p function on the molecular level may therefore not only further define its role in mitochondria, but may also provide intriguing novel clues on the mechanism of chaperonin action in general.

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