The Saccharomyces cerevisiae TCM62 Gene Encodes a Chaperone Necessary for the Assembly of the Mitochondrial Succinate Dehydrogenase (Complex II)*

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The assembly of the mitochondrial respiratory chain is mediated by a large number of helper proteins. To better understand the biogenesis of the yeast succinate dehydrogenase (SDH), we searched for assembly-defective mutants. SDH is encoded by the SDH1, SDH2, SDH3, and SDH4 genes. The holoenzyme is composed of two domains. The membrane extrinsic domain, consisting of Sdh1p and Sdh2p, contains a covalent FAD cofactor and three iron-sulfur clusters. The membrane intrinsic domain, consisting of Sdh3p and Sdh4p, is proposed to bind two molecules of ubiquinone and one heme. We isolated one mutant that is respiration-deficient with a specific loss of SDH oxidase activity. SDH is not assembled in this mutant. The complementing gene, TCM62 (also known as SCYBR044C), does not encode an SDH subunit and is not essential for cell viability. It encodes a mitochondrial membrane protein of 64,211 Da. The Tcm62p sequence is 17.3% identical to yeast hsp60, a molecular chaperone. The Tcm62p amino terminus is in the mitochondrial matrix, whereas the carboxyl terminus is accessible from the intermembrane space. Tcm62p forms a complex containing at least three SDH subunits. We propose that Tcm62p functions as a chaperone in the assembly of yeast SDH.

The respiratory chain of the mitochondrial inner membrane is typically composed of four multisubunit enzymes (complexes I–IV) and the ATP synthase (complex V). In Saccharomyces cerevisiae, the succinate dehydrogenase (SDH) or complex II subunits are encoded by the SDH1, SDH2, SDH3, and SDH4 genes. The four SDH subunits are as follows: a large flavoprotein (Sdh1p) subunit of 67 kDa to which is covalently attached an FAD cofactor (3), an iron-sulfur subunit (Sdh2p) of 28 kDa that contains three iron-sulfur clusters (4), and two small hydrophobic membrane subunits of 16.7 (Sdh3p) and 16.6 (Sdh4p) kDa (5, 6). The two small subunits serve as membrane anchors and are thought to contain two ubiquinone-binding sites and a b-type heme (7, 8).

A large number of gene products are necessary for the biogenesis of the respiratory chain. It is a complex process involving the expression of two genomes and the transport of hundreds of proteins into the organelle (9, 10). In addition to the many nuclear and to the seven (in yeast) mitochondrially encoded subunits of the five complexes, many gene products are necessary to replicate and express the mitochondrial DNA. Perhaps more surprising are the long lists of genes required for the post-translational assembly of the respiratory chain and the ATP synthase (9, 11–13). Some of these, like the COX10 and COX11 gene products, are involved in heme synthesis for complex IV biogenesis (14, 15). The COX17 gene encodes a mitochondrial copper shuttle necessary for metal delivery to complex IV (16). The ABC1 gene is essential for the correct assembly of complexes II-IV, and a loss of Abc1p impairs electron transport in complex III (8, 17). The BCS1 gene product may be necessary for the formation or insertion of the active-site iron-sulfur cluster of the Rieske protein of complex III (18). The ATP10 gene is required for the assembly of the F0 moiety of the ATP synthase (19), whereas the assembly of the F1 portion depends on functional ATP11 and ATP12 gene products (20, 21). Clearly, the assembly of the mitochondrial respiratory chain is not a spontaneous event, but is mediated by a large number of accessory proteins.

In this work, we identify a gene (TCM62) whose product is specifically required for SDH biogenesis. Deletion of the TCM62 gene results in respiration deficiency. Tcm62p is a 64,211-Da (572 amino acids) membrane-spanning protein with 17.3% sequence identity to yeast hsp60. It is synthesized as a precursor and imported into mitochondria with the concomitant cleavage of the presequence. Tcm62p forms a complex with at least three of the SDH subunits. Overexpression of Tcm62p results in the production of intramitochondrial inclusion bodies that also trap SDH subunits. We propose that Tcm62p is a chaperone essential for SDH biogenesis.

EXPERIMENTAL PROCEDURES

Strains, Vectors, and Media—The S. cerevisiae strain MH125 (a, trp1, ura3-52, leu2-3,112, his3, his4, rme, p +) is the parent of the mutant tcm62 (tcm62-1). CAS1 (tcm62Δ::HIS3) was constructed by one-step gene replacement in the parent strain YPH500 (a, ura3-52, lys2-801apo, ade2-101res, trp1Δ3, his3Δ200, leu2Δ1; Stratagene, La Jolla, CA). Segregation analysis was performed by mating with YPH499 (a, ura3-52, lys2-801apo, ade2-101res, trp1Δ3, his3Δ200, leu2Δ1) and sporulation. The yeast media were used as follows:YPD, a complex medium containing 1% yeast extract, 2% peptone, 0.1% glucose, and 3% glycerol; YPD, containing 1% yeast extract, 2% peptone, and 2% glucose; YPD-0.6%, containing 1% yeast extract, 1% peptone, 0.6% glucose, 0.1% KH2PO4, 0.12% ammonium sulfate, pH 6.2; and YPDBP, containing 1% yeast extract, 2% peptone, 0.5% glucose, and 30 μM b-microsulfate, pH 7.0 (22). Other yeast and Escherichia coli strains as well as media have been described (23). The vectors pRS413, pRS414, pRS416, pRS423, and pBluescript 11 were obtained from Stratagene.

Mutant Isolation—MH125 was exposed to ultraviolet light of 254 nm to achieve ~50% survival (24). Between 200 and 300 survivors were plated on each of 100 YPDG plates and incubated at 30 °C for 5 days.

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The plates were overlaid with 67 mm KH\textsubscript{2}PO\textsubscript{4} and 0.1% 2,3,5-triphenyltetrazolium chloride, pH 7.0, in 1.5% agar (25). 124 small colonies that stained dark pink or reddish were picked onto YPD plates, grown at 30 °C for 24 h, and replicated onto YPDBP plates to detect acid secretion. Submitochondrial particles were isolated from 22 acid-producing strains grown in YPD-0.6% and analyzed for oxygen consumption with one of the following the substrates: succinate, NADH, or glycerol 1-phosphate (23).

**Cloning and Sequencing**—A yeast genomic library in the single-copy shuttle vector YCP50 was transformed into the mutant tcm62, and ~1000 uracil prototrophs were obtained (28). These were replicated onto minimal medium with glycerol as the sole carbon source; two transformants were capable of growth. The plasmid pTCM61-1 carries a 10-kb insert that was subcloned as a 2.5-kb BglII-ClaI fragment in the single-copy TRP1-selectable vector pRS414 to create the plasmid pTCM62-7. The same fragment was inserted into the vectors pRS416 and YEpPlac195 (27) to create the URA3-selectable single-copy plasmid pTCM62-21 and the multi-copy plasmid pTCM62-23, respectively. The copper-inducible expression plasmid pTCM62-25 was created by inserting the TCM62 open reading frame into pUCu (28). DNA sequence was determined by fluorescent dye-tagged dyeoxy chain termination on an Applied Biosystems Model 373A DNA sequencer.

**Construction of a Chromosomal Disruption Mutant**—A polymerase chain reaction with the oligonucleotides 5'-CCGACTCATGCTATACTCAGTTGTCGGGCTCTCCAAGATG-3' and 5'-AGTATCTGCTTAAGGGCTTTATCTAGGCTTTGTGCCGTTCAAGCCTCGTTGTCACATG-3' was performed to amplify the HIS3 gene from pRS413 and to flank it with TCM62 upstream and downstream sequences (underlined). The amplification product was transformed into YPH500, and HIS3 prototrophs were selected (29).

**Construction of Tagged Tcm62p**—A polymerase chain reaction with the oligonucleotide 5'-TACGTTCTGGGCTACACGTCATTCTCAGAAAATACGTTTGTTCACCGCCGGCTTGTGGCCTCAG-3' was performed to add the 10-residue c-myc proto-oncogene sequence (double-underlined) recognized by the monoclonal antibody 9E10 (30) to the carboxyl terminus of Tcm62p (underlined). The oligonucleotide incorporates 2 glycine residues between the carboxyl terminus of Tcm62p (underlined) and c-myc (double-underlined) recognized by the monoclonal antibody 9E10 (31). The plasmid pTCM62-myct was digested with NaeI (introduced during the myc tagging), and a fragment containing three tandem copies of the HA1 epitope recognized by the monoclonal antibody 12CA5 (31). The plasmid pTCM62-myct encoding the tagged protein was transformed into YPH500, and lysed through a French pressure cell to prepare submitochondrial membranes (23). Succinate and NADH oxidase activities were measured in a Clarke-type electrode. Four mutants displayed a specific loss of succinate oxidase activity and were analyzed in more detail. Three of the isolated mutants were identified as SDH1 mutants by complementation analysis. The mutant tcm62 defines a complementation group distinct from the SDH1, SDH2, SDH3, and SDH4 genes.

As shown in Fig. 1A, mitochondrial membranes from the wild-type strain MH125 displayed considerable malonate-sensitive succinate oxidase activity as well as antimycin A-sensitive NADH oxidase activity. In contrast, tcm62 membranes had little succinate oxidase activity, but retained high levels of NADH oxidase activity (Fig. 1B). Since succinate and NADH oxidase activities use a series of common electron carriers following the reduction of ubiquinone, the specific loss of succinate oxidase activity in tcm62 indicates a defect in the SDH component of the respiratory chain.

**Properties of the tcm62 Mutant**—Segregation analysis after mating, sporulation, and dissection of ascie revealed that the respiration-negative phenotype segregated 2:2 (data not shown) and thus is due to a single recessive nuclear mutation. The tcm62 mutant is unable to grow on minimal glycerol medium, but displays some growth on a rich glycerol medium. tcm62 submitochondrial membranes display an almost complete loss of all SDH-dependent activities, including succinate oxidase, succinate-cytochrome c reductase, succinate-ubiquinone reductase, and succinate-dichloroflenidophenol reductase activities (data not shown). There were variable losses of NADH oxidase, NADH-cytochrome c reductase, glycerol-phosphate oxidase, and glycerol-cytochrome c reductase activities in different preparations. To further characterize the function of the TCM62 gene in a non-mutagenized background and possibly eliminate the variability of respiratory activity levels between preparations, we decided to clone the wild-type gene and to create a null allele by a one-step gene disruption.

**Cloning and Sequencing of TCM62**—The TCM62 gene was cloned by complementation of the tcm62 mutant (26). Two respiratory-competent and auxotrophic prototrophic clones were obtained. Both complementing plasmids were isolated, found to be identical by restriction analysis, and named pTCM62-1. The gene was localized by subcloning different regions of the pTCM62-1 insert into pRS414, and the resultant plasmids were tested for their ability to complement the tcm62 mutant. The complementing activity could be minimally subcloned as a
2.5-kb BglII-ClaI fragment, creating the plasmid pTCM62-7. Sequence was determined from both strands for the entire open reading frame and flanking regions of the 2.5-kb BglII-ClaI fragment. The fragment contained a single long open reading frame that was identified as the TCM62 gene based on the subcloning results and the targeted gene disruption (see below). The TCM62 gene sequence we determined is identical to a chromosome II sequence encoding the hypothetical protein SCYBR044C (GenBank™ accession number Z35913). The open reading frame predicts a protein of 572 amino acids with an Mr of 64,211. TCM62 does not encode a known SDH subunit. Rather, Tcm62p is most closely related to the hsp60 family of molecular chaperones called chaperonins (34–36). An alignment of Tcm62p with Culicoides variipennis hsp60, yeast hsp60, and E. coli GroEL is displayed in Fig. 2. The Tcm62p sequence is 17.7, 17.3, and 16.0% identical to these proteins, respectively (calculated as the number of identities over the number of residues plus the number of gaps). The sequence similarities, which are distributed throughout the entire protein, are 53.4, 54.4, and 53.7%, respectively. For comparison, yeast hsp60 and GroEL have 51.7% identical residues between them. The Tcm62p Blast2 score against C. variipennis hsp60 is 2.3 x 10^-15, a score that justifies the inclusion of Tcm62p as a member of the hsp60 family of proteins. Residues 469–491 are predicted to be membrane-spanning (Fig. 3); the comparable regions in the chaperonins have additional charged amino acids (Fig. 2).

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Targeted Gene Disruption of TCM62—To characterize the phenotype of a null allele of TCM62 and to confirm that the pTCM62-1 plasmid is responsible for complementation of the respiratory defect of the mutant tcm62, we created a null allele by targeted gene disruption. We utilized a polymerase chain reaction approach where the oligonucleotides contain two regions, one that directs homologous recombination at the target locus and a second region that allows for amplification of a selectable marker (29). The first oligonucleotide consists of the sequence from 244 to 22 (where the A of the first codon is position 11) of TCM62 followed by 20 bases located upstream of the HIS3 promoter. The second oligonucleotide consists of the sequence complementary to positions 1737–1698 (where the T of the amber codon is position 1717) followed by 20 bases located downstream of the HIS3 gene. The primers were used to amplify a 1-kb portion of the plasmid pRS413 encoding the HIS3 gene, thus flanking it with 40 and 43 bases of TCM62 sequences. The polymerase chain reaction product was transformed directly into YPH500 bearing the his3::HIS3 allele, a complete deletion of the HIS3 gene, and transformants were selected by complementation of the histidine auxotrophy. Twenty transformants were obtained; two were found to contain the desired gene replacement by polymerase chain reaction and Southern blot analyses (data not shown). The successful isolation of a null mutant in a haploid strain indicates that TCM62 is not essential for viability. The disruption mutant CAS1, like tcm62, is respiration-deficient; it does not grow on minimal medium containing glycerol, but does show slow growth on a rich glycerol medium.

When we mated CAS1 with YPH499 and sporulated the diploid, we found that in 10 of 11 dissected tetrads, histidine prototrophy and respiration deficiency co-segregated, indicating that the gene replacement is the cause of the respiration deficiency. When tcm62-1 and tcm62::HIS3 were combined in a diploid, the respiration deficiency persisted, indicating
that the two mutations belong to the same complementation group. The tcm62Δ2::HIS3 mutation was also complemented by the plasmid pTCM62-7.

**TCM62 Is Necessary for SDH Function**—Submitochondrial membranes from the parent strain YPH500, CAS1, CAS1 with the single-copy plasmid pTCM62-21, or CAS1 with the multicy copy plasmid pTCM62-23 were prepared from cultures grown for 46 h on YPD at 30 °C (37). In all cases, >80% of the cells retained the plasmid and were p⁺. The membranes were tested for the levels of several respiratory chain activities (Table I). In CAS1, the three SDH-dependent activities we measured (succinate oxidase, succinate-cytochrome c reductase, and succinate-phenazine methosulfate reductase) were all reduced at least 45-fold, whereas the glycerol-phosphate dehydrogenase and NADH dehydrogenase-dependent activities were reduced, at most, 4-fold. Complementation of the tcm62Δ2::HIS3-induced respiratory defect by pTCM62-21 or pTCM62-23 considerably restored SDH-dependent activities, but never to wild-type levels. Indeed, the multicopy plasmid was, in general, less effective at restoring the activities. In the cases of the NADH oxidase, glycerol-phosphate oxidase, and NADH-cytochrome c reductase activities, the presence of the plasmid and presumably overexpression of Tcm62p actually reduced activity levels to below those of the mutant without plasmid. This observation remains to be explained. We conclude that Tcm62p is absolutely required for the production of functional SDH, but that the loss of Tcm62p or its expression at altered levels also affects the assembly of other respiratory protein complexes, although to a much lesser extent.

When we analyzed CAS1 mitochondrial proteins by Western blot analysis, we observed the complete absence of the Sdh2p subunit (Fig. 4D, lane 1), but the levels of the Cox4p subunit of the cytochrome c oxidase (Fig. 4A, lane 1), the intermembrane space-localized cytochrome b₅₆₇ (Fig. 4B, lane 1), and the β-subunit of the ATP synthase (Fig. 4C, lane 1) were unaffected when compared with the parent strain (lanes 2). As expected, Sdh1p is also missing in CAS1 mitochondria (data not shown); when one SDH subunit is missing, all others are apparently also lost (3, 23, 32). These data confirm the specific requirement for Tcm62p function in SDH biogenesis.

**Tcm62p Is a Mitochondrial Protein**—The amino-terminal 35 residues, being rich in basic amino acids and devoid of acidic ones, are suggestive of a mitochondrial targeting sequence. *In vitro* synthesized precursor protein (Fig. 5, lane 1) was imported into isolated yeast mitochondria. Tcm62p precursor bound to mitochondria (lanes 2 and 5) and could be imported in an energy-dependent manner (lane 3 versus lane 6) to a protease-protected location (lane 3) that is made accessible by the addition of detergent (lane 4). Import was accompanied by a proteolytic cleavage event to a faster migrating species (lanes 2 and 3). The consequences of the chaperonin 60 proteins are usually between 25 and 35 residues in length; the size change upon the maturation of the imported Tcm62p is consistent with a presequence of this length.

**Tcm62p Is a Membrane Protein**—Examination of the Tcm62p amino acid sequence using a variety of predictive algorithms, including that developed by Kyte and Doolittle (38), suggested that residues 469–491 form a transmembrane segment (Fig. 3). To test this prediction, we examined the solubility properties of the HA1-tagged protein. Tcm62p could not be released from mitochondria by washes with 0.5 M NaCl, 20 mM EDTA, or 8 M urea, suggesting that the protein is anchored by non-ionic interactions (Table II). Washing mitochondria with 0.1 M Na₂CO₃, pH 11.5, completely solubilized Tcm62p, whereas a similar wash at pH 10.5 was only partially effective at solubilizing the protein. The results are intermediate between the truly integral protein porin, which is not re-

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**Fig. 3. Hydropathy analysis of Tcm62p.** The algorithm of Kyte and Doolittle (38) using a window of 11 residues was used to analyze the hydrophy. Hydrophobic segments have positive values.

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**Table I** Respiratory chain activities of mitochondrial membranes

|                   | YPH500 | CAS1⁺ | pTCM62–21⁺ | pTCM62–23⁺ |
|-------------------|--------|-------|------------|------------|
| Succinate oxidase | 73     | 2.2   | 53         | 27         |
| Glycerol-P oxidase| 22     | 41    | 50         | 13         |
| NADH oxidase      | 172    | 67    | 35         | 13         |
| Succinate-cytochrome c reductase | 140 | 1     | 80         | 59         |
| Glycerol-P-cytochrome c reductase | 40.6 | 58    | 88         | 76         |
| NADH-cytochrome c reductase | 228 | 98    | 111        | 56         |
| Succinate-FMS reductase | 139 | 0.4   | 35         | 68         |
| Glycerol-P-PMS reductase | 33.9 | 24    | 39         | 65         |

a Activities are expressed as percentages of YPH500.

b Activities are expressed as ng atoms of oxygen/min/mg of protein.

c Activities are expressed as μmol of cytochrome c reduced per min/mg of protein.

d FMS, phenazine methosulfate.

— Activities are expressed as μmol dichlorophenol indophenol reduced per min/mg of protein.

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**Fig. 4. CAS1 mitochondria are specifically depleted of SDH.** CAS1 (lanes 1) or YPH499 (lanes 2) mitochondrial proteins (20 μg/lane) were analyzed by Western blot analysis using antisera directed against Cox4p (A), cytochrome b₅₆₇ (B), the β-subunit of the ATP synthase (C), and Sdh2p (D). All antisera were diluted 5000-fold. The positions of the relevant proteins are indicated by arrows. The size markers are indicated on the left in kDa.

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2 E. Dibrov, S. Fu, and B. D. Lemire, unpublished observations.
leased at pH 11.5, and Sdh2p, which is a peripheral protein that is released with urea and at high pH. In practice, Tcm62p can only be efficiently solubilized with non-ionic detergents such as lauryl maltoside or Triton X-100. The partial solubilization by Na₂CO₃ may reflect the presence of charged amino acids (Glu-486 and Lys-490) in the potential transmembrane segment; these may destabilize membrane attachment at high pH when only the glutamic acid is charged, but form a salt bridge compatible with membrane integration at neutral pH.

If Tcm62p is a transmembrane protein, it should contain a domain in the intermembrane space. HA1-tagged Tcm62p was inaccessible to added proteinase K in isolated mitochondria (Fig. 6A, lane 1). Upon disruption of the outer membrane by hypotonic shock (lane 2) or disruption of both mitochondrial membranes by sonication (lane 3), the HA1 tag could be completely digested, indicating that it is the outer membrane that protects the tag from digestion in intact organelles. When the accessibilities of the matrix-localized control proteins Sdh2p, hsp60, and F₁β were examined, these were found to be resistant to proteinase in intact or outer membrane disrupted mitochondria (Fig. 6, B–D, lanes 1 and 2, respectively), but susceptible to digestion when the inner membrane was opened (lanes 3). When the accessibility of the control intermembrane space protein cytochrome b₂ was examined, it paralleled that of HA1-tagged Tcm62p; it became mostly accessible when the outer membrane was broken, but when the inner membrane was still intact (Fig. 6E, lane 2). These results place the Tcm62p carboxyl terminus in the intermembrane space. Presupersence cleavage upon import is performed by a matrix-localized metalloproteinase (39), indicating that the amino terminus is in that compartment. Tcm62p thus contains at least one membrane-spanning domain crossing the mitochondrial inner membrane.

**Fig. 5. Tcm62p is imported into isolated mitochondria.** Radio-labeled Tcm62p was produced in vitro and imported into isolated mitochondria (3). Lane 1, 10% of the amount of lysate added to each import reaction; lane 2, energized mitochondria incubated with Tcm62p precursor; lane 3, same as lane 2, but after treatment with 250 μg/ml proteinase K; lane 4, same as lane 3, but the mitochondria were disrupted by the addition of 1% Triton X-100 before treatment with proteinase; lane 5, mitochondria de-energized with 10 μM valinomycin and incubated with Tcm62p precursor; lane 6, same as lane 5, but treated with proteinase. The positions of size markers are indicated on the right in kDa. p, precursor form; m, mature form. Note that the precursor form of Tcm62p migrated somewhat faster in lanes 2 and 5 because of the presence of bovine serum albumin (69 kDa), used to maintain the import competence of mitochondria during storage at −70 °C, as compared with lane 1, which did not have added mitochondria.

**Tcm62p Interacts with SDH—**The sequence similarities between Tcm62p and hsp60 proteins led us to hypothesize that Tcm62p physically interacts with one or more of the SDH subunits. We solubilized mitochondrial proteins containing HA1-tagged Tcm62p and separated them by blue native gel electrophoresis. Blue native gel electrophoresis is a separation technique that uses nondenaturing nonionic detergents to solubilize membrane protein complexes and to separate them on the basis of size (6, 40, 41). SDH subunits migrate in three separate complexes: a tetrameric complex consisting of Sdh1p-Sdh4p, a faster migrating species that we have postulated to represent an Sdh1p-Sdh2p dimer, and a slower migrating species that may correspond to homo-oligomers of SDH or to SDH associated with other respiratory chain complexes (6, 41). In the minigel system used here, only the two slower migrating species were detected. Proteins resolved by blue native gel electrophoresis in the first dimension and denaturing Tricine-SDS gel electrophoresis in the second dimension were electrophoretically transferred to Immobilon-P membranes (Millipore Corp.) for Western blot analysis with antibodies directed against the HA1 epitope or SDH subunits (Fig. 7). In Fig. 7A, a single species of Tcm62p was detected in membranes from CAS1 transformed with pTCM62-HA, whereas two species of SDH (arrows 3 and 4) were detected with the antisera directed against Sdh1p, Sdh2p, or Sdh3p. In YPH500 membranes (Fig. 7B), which do not contain HA1-tagged Tcm62p, only the two forms of SDH were detected. Thus, HA1-tagged Tcm62p co-migrated with the slower migrating SDH species (arrow 3), suggesting a physical association between Tcm62p and SDH. Surprisingly, when an SDH1 knockout strain, sdh1L6 (42), or an SDH2 knockout strain (data not shown) was transformed with pTCM62-HA, Tcm62p migrated more slowly during blue native gel electrophoresis and had an estimated mass of 450 kDa (Fig. 7C). As markers, we reprobed the membrane with antisera against the β-subunit of the ATP synthase and against the Cox4p subunit of complex IV, which have masses of 600 and 200 kDa, respectively (40).

When myc-tagged Tcm62p was expressed under control of the copper-induced CUP1 promoter, we found that not all of the Tcm62p could be solubilized by nonionic detergents. Furthermore, we also noticed that Sdh2p solubilization became incomplete. We suspected that Tcm62p overexpression resulted in insoluble protein. To test this hypothesis, we isolated mitochondria from YPH500 or from YPH500 transformed with pRSCu62-myc. Mitochondrial proteins were solubilized and fractionated following a protocol designed to isolate inclusion bodies (43). In Fig. 8A, myc-tagged Tcm62p was easily detected in the pRSCu62-myc-transformed YPH500 mitochondria (lane 2), but was absent in YPH500 mitochondria (lane 1), as expected. Almost all of the myc-tagged Tcm62p was found to be detergent-insoluble when the pellets of the inclusion body preparations were examined (lane 4), confirming that overexpressed Tcm62p aggregates after its import into mitochondria. Western blot analysis with the anti-Sdh2p (Fig. 8B) or anti-

**Table II**

| Protein | pH 11.5 | pH 10.5 | EDTA | NaCl | Urea |
|---------|---------|---------|------|------|------|
|         | S | P | S | P | S | P | S | P |
| Tcm62p  | + | − | ± | + | + | + | + | + |
| Sdh2p   | + | + | + | + | + | + | + | + |
| Porin   | − | + | ND | ND | − | + | + | ± |

* Mitochondria prepared from CAS1 carrying pTCM62-HA were treated with 0.1 M Na₂CO₃, pH 11.5; 0.1 M Na₂CO₃, pH 10.5; 20 mM EDTA; 0.5 mM NaCl; or 8 M urea for 30 min on ice. Insoluble material was pelleted by centrifugation for 35 min at 390,000 × g. Supernatants (S) and pellets (P) were probed by Western blot analysis with anti-HA1, anti-SDH2p, and anti-porin antibodies.

* ND, not done.
Sdh1p (Fig. 8C) antisera revealed that these SDH subunits were also overexpressed in pRSCu62-myc-transformed YPH500 (lanes 2) as compared with YPH500 (lanes 1) and were largely associated with the inclusion body pellets (lanes 4), although portions were solubilized (lanes 6). Neither the Sdh2p nor the Sdh1p subunit was found in the inclusion body pellets of YPH500 mitochondria (lanes 3), indicating they are completely solubilized (lanes 5). These observations suggest that overexpressed Tcm62p traps SDH subunits with it as it forms insoluble aggregates. The inclusion bodies do not trap all proteins since hsp60 is absent and the β-subunit of the ATP synthase is detected in only trace amounts (data not shown).

**DISCUSSION**

Hsp60 proteins belong to a subclass of molecular chaperones called chaperonins and are found in all bacteria, mitochondria, and plastids (34, 36, 44). Yeast hsp60 exists as an oligomer of 14 subunits and mediates the assembly of oligomeric complexes (45) and the folding of some newly imported monomeric proteins (46). The folding mechanism involves the hydrolysis of ATP (47) and the cooperation of a smaller protein of the chaperonin 10 class (48, 49).

We have isolated a gene (TCM62) that encodes a member of the hsp60 family of mitochondrial chaperones. Tcm62p has 16 and 17.3% sequence identities, distributed along the entire protein sequence, to E. coli GroEL and yeast hsp60, respectively (Fig. 2). The chaperonins contain several motifs that are highly conserved. One such motif is associated with ATP binding and includes the residues of the phosphate-binding loop DGGTTT (residues 87–91, numbering and sequence are from GroEL; see Fig. 2) (50, 51). Asp-87 is essential for the ATPase activity of GroEL (52). In Tcm62p, the corresponding sequence reads DNLTAT (residues 121–126; see Fig. 2). Other motifs near the ATP-binding site are TITZDG (residues 48–53) and GGG (residues 414–416); in Tcm62p, the first motif is missing, whereas the second is GYG (residues 446–448) (53). If Tcm62p does bind ATP, the interactions between the nucleotide and the protein will be significantly altered from those seen in the GroEL structures (50, 54). Experiments are in progress to determine whether Tcm62p binds ATP and is an ATPase, but ATP hydrolysis is not a universal property of chaperones (44).

What is the function of Tcm62p? Tcm62p is less abundant than SDH since the majority of SDH migrates as a tetramer not associated with Tcm62p (Fig. 7, A and B). This is consistent with a catalytic chaperone function for Tcm62p. In a wild-type strain, Tcm62p is found exclusively associated with SDH when mitochondrial proteins are solubilized and resolved by blue native gel electrophoresis (Fig. 7A). In the absence of SDH, we detected an oligomeric form of Tcm62p (Fig. 7C). We do not know the composition of this oligomer, but it tempting to speculate that Tcm62p exists as ring-like heptamers, although un-
like other chaperones, these would be membrane-associated.

The amount of Tcm62p oligomer in an SDH1 disruption mutant is much more than in an SDH2 disruption mutant (data not shown), possibly suggesting a primary stabilizing interaction between Tcm62p and Sdh2p. Overexpression of Tcm62p results in the production of inclusion bodies containing Sdh2p and Sdh1p subunits (Fig. 8). Interactions between SDH and Tcm62p may be through the iron-sulfur subunit since there appears to be a particular enrichment of Sdh2p in the aggregates (Fig. 8B, lane 4). Sdh1p is not as enriched and may be in the inclusion body through its interactions with Sdh2p, or it may be underrepresented due to degradation. The most prominent features of the Sdh2p subunit are its three iron-sulfur clusters, and Tcm62p may be involved in the formation or insertion of the clusters into apo-Sdh2p. Alternatively, Tcm62p may stabilize the holo-Sdh2p subunit until it can form a stable complex with the Sdh1p subunit.

The loss of Tcm62p function greatly affects SDH, with only minor effects on other respiratory chain components. Tcm62p would not be unique in having a single substrate (44). Why are the levels of other respiratory chain complexes affected by mutations in TCM62? Tcm62p may have a facilitating function in the assembly of many complexes, but only SDH absolutely requires it. Other complexes may possess alternate assembly pathways or may be able to assemble spontaneously, although less efficiently. It is interesting to note that the Sdh2p subunit contains the only [3Fe-4S] in the respiratory chain and that this cluster type may have more stringent assembly requirements. Alternatively, the decreases in other enzymatic activities may be a secondary effect due to the absence of respiration.

The TCM62 gene has a number of parallels with the ABC1 gene, which is required for complex III assembly (17). First, the Abc1p sequence is weakly related to the chaperones (17). Second, both Abc1p and Tcm62p are postulated to be assembly factors that have some specificity for a particular respiratory chain complex. Third, the TCM62 and ABC1 deletions result in respiratory chain defects that may be explained by either direct or indirect effects of Abc1p and Tcm62p on the assembly of several complexes. Brasseur et al. (8) favor the explanation that the pleiotropic effects of an ABC1 deletion are due to indirect effects arising from interactions of the other respiratory complexes with an altered complex III. There is evidence for a close interaction between complexes II and III since some mutations in complex III subunits interfere with SDH function (56, 57). The tcm62a::HIS3 mutation results in an almost complete loss of SDH-dependent activities, with little or no loss of complex III function as judged by the NADH-cytochrome c reductase activities (Table I). Similarly, SDH structural gene mutations do not affect complex III (6, 23). Thus, complexes II and III can function independently under these circumstances. The tcm62a::HIS3 mutation does partially impair the assembly or function of the glycerol-phosphate dehydrogenase and cytochrome c oxidase complex. A more detailed characterization of the role of Tcm62p in the biogenesis of SDH and the yeast respiratory chain is in progress.