The Functional Interaction of Mitochondrial Hsp70s with the Escort Protein Zim17 Is Critical for Fe/S Biogenesis and Substrate Interaction at the Inner Membrane Preprotein Translocase*

Ilka Lewrenz†, Nicole Rietzschel‡, Bernard Guiard§, Roland Lill¶, Martin van der Laan** and Wolfgang Voos††

From the †Institut für Biochemie und Molekularbiologie, Universität Bonn, Nussallee 11, D-53115 Bonn, Germany, the ‡Institut für Zytobiologie und Zytopathologie, Universität Marburg, Robert-Koch Strasse 6, 35032 Marburg, Germany, the §Centre de Génétique Moléculaire, Laboratoire Propre du CNRS Associé à l’Université Pierre et Marie Curie, 91190 Gif-sur-Yvette, France, the ¶Institut für Biochemie und Molekularbiologie (ZBMZ), Universität Freiburg, Stefan-Meier-Strasse 17, 79104 Freiburg, Germany, and the **BIOSS Centre for Biological Signalling Studies, Universität Freiburg, 79104 Freiburg, Germany

Background: Zim17 represents a Hsp70-interacting protein residing in the mitochondrial matrix compartment.

Results: Conditional zim17 mutants show functional defects in essential mtHsp70 activities, biosynthesis of Fe/S clusters, and interaction with newly imported substrate proteins.

Conclusion: Zim17 exerts a direct regulative function on Hsp70 independent of its aggregation-protective role.

Significance: Identification of novel co-chaperone mechanism unravels complex functional regulation of Hsp70 chaperones.

The yeast protein Zim17 belongs to a unique class of co-chaperones that maintain the solubility of Hsp70 proteins in mitochondria and plastids of eukaryotic cells. However, little is known about the functional cooperation between Zim17 and mitochondrial Hsp70 proteins in vivo. To analyze the effects of a loss of Zim17 function in the authentic environment, we introduced novel conditional mutations within the ZIM17 gene of the model organism Saccharomyces cerevisiae that allowed a recovery of temperature-sensitive but respiratory competent zim17 mutant cells. On fermentable growth medium, the mutant cells were prone to acquire respiratory deficits and showed a strong aggregation of the mitochondrial Hsp70 Ssq1 together with a concomitant defect in Fe/S protein biogenesis. In contrast, under respiring conditions, the mitochondrial Hsp70s Ssc1 and Ssq1 exhibited only a partial aggregation. We show that the induction of the zim17 mutant phenotype leads to strong import defects for Ssc1-dependent matrix-targeted precurser proteins that correlate with a significantly reduced binding of newly imported substrate proteins to Ssc1. We conclude that Zim17 is not only required for the maintenance of mtHsp70 solubility but also directly assists the functional interaction of mtHsp70 with substrate proteins in a J-type co-chaperone-dependent manner.
import of nucleus-encoded preproteins into the mitochondrial matrix. Scs1 is recruited to the inner membrane import channel, the TIM23 complex, by the scaffold protein Tim44, where it interacts with incoming preproteins (2, 11, 12). In an ATP-dependent reaction, Scs1 drives the vectorial translocation of the precursor polypeptide through the import channel (13). During import, Scs1 function is assisted by Pam18, a class III J-protein that consists essentially of a membrane-attached J-domain (14). Scs1 also plays a role in folding of unfolded or denatured polypeptides in the matrix compartment in cooperation with the class I J-protein Mdj1 (15, 16). Like all class I J-proteins, Mdj1 is able to bind directly to substrate proteins and delivers them to Scs1. The second, less abundant mtHsp70 protein, Ssq1, fulfills a highly specialized task in the mitochondrial Fe/S-cluster assembly machinery (17). The only known substrate of Ssq1 is Isu1, a scaffold protein on which the Fe/S-cluster is synthesized de novo before it is transferred to the respective apoenzyme (18). During Fe/S-cluster biogenesis, the class III J-protein Jac1 specifically binds to holo-Isu1 through a region in its C-terminal domain and delivers it to the Hsp70 chaperone (19). Because Ssq1 and Jac1 are highly selective for Isu1, this system appears to be far more specific than the Ssc1 chaperone system. Although the functions of Ssc1 and Ssq1 are well characterized, little is known about Ecm10, and its precise function remains to be determined.

Recently, a novel type of Hsp70 co-chaperone that is characterized by a central zinc-binding domain consisting of two CXXC motifs flanked by several β-sheets has been identified (20). The yeast family member has been named Zim17 (zinc finger motif protein of 17 kDa), Tim15 (translocase of the inner membrane component of 15 kDa), or Hsp1 (Hsp70 escort protein). Based on a highly conserved Asp-Asn-Leu motif close to the zinc-binding residues, the cysteine-rich region has been newly classified as a DNLZ-type zinc finger (21). DNLZ proteins are conserved in chloroplasts and mitochondria of all bacteria or archaea (22–24). On the basis of its structural properties, it was initially proposed that Zim17 supplements the activity of class II or III J-proteins by providing a zinc finger domain (22). However, recent experiments have focused on a stabilizing function that Zim17 exerts on Hsp70 chaperones. Yeast Zim17 has been shown to interact with both Ssc1 and Ssq1 in the mitochondrial matrix. The binding of Zim17 to mtHsp70s occurs most likely in the absence of nucleotides (23), and it has been proposed to stabilize this aggregation-prone conformational state of the chaperone. Indeed, a complete loss of Zim17 in yeast deletion mutants (zim17Δ) resulted in the aggregation of the mitochondrial chaperones Ssc1 and Ssq1. Concomitantly, zim17Δ cells showed severe phenotypic defects like the inability to grow at elevated temperatures and a high tendency to generate the respiratory deficient petite phenotype (22, 23, 25, 26). This phenotype has been explained by the long term accumulation of secondary defects that are related to a loss of chaperone activity.

However, due to this pleiotropic phenotype, it has been difficult to define the primary effects of a loss of Zim17 function in the cellular context. Most biochemical data on the effect of Zim17 on Hsp70 aggregation have been obtained by in vitro experiments employing purified components or using heterologous expression systems in bacteria. Such conditions do not necessarily reflect the behavior of proteins in their natural environment. In this work, we utilized novel temperature-sensitive mutations in ZIM17 to obtain direct insights into its biochemical function in the authentic cellular environment and to directly assess the consequences of a loss of Zim17 independent of accumulative secondary defects. We pursued several cell biological and biochemical approaches to clarify the precise nature of the Zim17-dependent mtHsp70 functions both under normal and non-permissive conditions. Our results indicate that the functional interaction of Zim17 with its mtHsp70 partners is not restricted to the prevention of their aggregation but rather exhibits a more complex and direct influence on the enzymatic properties of its cognate Hsp70 chaperone partner.

**EXPERIMENTAL PROCEDURES**

Genomic Integration and Random Spore Analysis—The WT ZIM17 gene and the mutant alleles zim17-3a and zim17-3b were fused to the LEU2 gene and inserted into the genome of Saccharomyces cerevisiae strain YPH499 through homologous recombination, replacing the endogenous ZIM17 gene. The mutant zim17-3a gene sequence was amplified from the plasmid pFLzim17-Ts3-CEN obtained from the yeast strain BGY-Fomp3-C1 (26). The novel mutant zim17-3b was generated by site-directed mutagenesis using PCR for the replacement of the serine residue at position 79 of mutant zim17-3a with an asparagine.

Genomic integrants were crossed with the WT strain BY4742, and diploid cells were selected by growth on minimal dropout medium without leucine and tryptophane. Sporulation was carried out at 25 °C for 5–10 days in sporulation medium (1% potassium acetate, 0.1% yeast extract, 77 mg/liter CSM-Ura (MP Biomedicals), 50 mg/liter uracil) until a sporulation rate of 50% or higher was reached. Tetrades were treated with 50 mg/ml zymolase at 30 °C. AscI and remaining diploid cells were disrupted with glass beads, and the remaining spores were plated out on YPD medium (1% yeast extract, 2% bacto-peptone, 2% glucose, pH 5.0). A random spore analysis was carried out by replica plating to drop-out media lacking leucine, tryptophane, or both. Temperature sensitivity and respiratory competence of spores carrying the mutant zim17 alleles were assayed by subjection to non-permissive temperature in both fermentable (YPD) and non-fermentable (YPG) conditions. Details of the yeast strains used in this study are listed in Table 1.

Isolation of Mitochondria and in Vitro Import Reaction—Yeast strains were grown in YPG to early log phase (A600 = 0.6), separated into two halves, and further grown in fresh YPG medium for 16 h at 25 °C or for 16 h at 37 °C to induce the temperature-sensitive phenotype. Mitochondria were isolated from cultures that had reached the mid-log phase (A600 = 1–1.5) by differential centrifugation as described (27) and were stored in SEM buffer (250 mM sucrose, 1 mM EDTA, and 10 mM MOPS, pH 7.2) at −80 °C.

The radiolabeled precursor protein Su9(86)-DHFR was imported into isolated yeast mitochondria according to established procedures (28). For the analysis of the inwardly directed translocation activity of Ssc1 (‘pulling assay’), radiolabeled b(167)4-DHFR was arrested as translocation intermediates...
with an import in the presence of the dihydrofolate reductase (DHFR) ligand methotrexate (MTX) at 5 μM. The precursors were pretreated with MTX, and the import reaction was performed for 15 min at 25 °C. The import was terminated by the addition of valinomycin, and samples were treated with proteinase K (50 μg/ml) for 15 min. After reisolation of mitochondria, proteins were separated by SDS-PAGE.

To evaluate the folding activity of Ssc1 in the mitochondrial matrix, radiolabeled, urea-denatured Su9(86)-DHFR was imported into isolated mitochondria as described above. Mitochondria were reisolated and lysed in lysis buffer (200 mM KCl, 30 mM Tris–HCl, pH 7.5, 5 mM EDTA, and 0.5% Triton X-100) containing 180 μg/ml proteinase K. The digestion was stopped after 10 min at 0 °C by the addition of 4 mM PMSF. TCA-precipitated proteins were separated by SDS-PAGE, and protease-resistant DHFR was quantified by autoradiography.

**Co-Immunoprecipitation of Imported Proteins**—Co-immunoprecipitation of imported proteins with mtHsp70 using immobilized antibodies against Ssc1 were performed as described (28). To examine the interaction of Ssc1 with Zim17, radiolabeled Zim17–WT, -3a, -3b, and Mdh1 were imported into isolated WT mitochondria for 20 min at 25 °C. After the import reaction was terminated, mitochondria were further incubated at 25 °C for 30 min prior to the immunoprecipitation.

To assess the interaction of Ssc1 with newly imported substrates, radiolabeled Su9(86)-DHFR was urea-denatured as described above, imported into mitochondria for 5 min, and subsequently subjected to immunoprecipitation. Samples were analyzed by digital autoradiography, and the amounts of radiolabeled proteins co-precipitated with Ssc1 were quantified. Due to the differing import efficiencies in the zim17 mutant mitochondria, the amounts of co-precipitated Su9(86)-DHFR were set in relation to the total amounts of imported protein. Because both zim17 mutants displayed differing levels of soluble Ssc1 and a partial Ssc1 aggregation phenotype at non-permissive temperature, the relative amounts of co-precipitated substrate proteins were additionally normalized to the precipitation efficiency.

**Binding of Ssc1 to ATP- and Reduced Carboxymethylated α-Lactalbumin (RCMLA)-Sepharose**—RCMLA was prepared from lactalbumin (Sigma) as described (29) and coupled to cyanobromide-activated Sepharose (GE Healthcare). Isolated mitochondria were suspended in import buffer and preincubated in presence of 2 mM ATP for 15 min at 25 °C. Mitochondria were reisolated and lysed, and protein extracts were incubated with ATP-agarose or RCMLA-Sepharose, as described (28). Samples were analyzed by SDS-PAGE and immunoblot.

**Aggregation Assay**—Mitochondria were pelleted and lysed with 1 μl of lysis buffer (200 mM KCl, 30 mM Tris, pH 7.5, 5 mM EDTA, 0.5% Triton, 0.5 mM PMSF, and protease inhibitors) per μg of mitochondria. A 25-μl total sample was taken, and the remaining lysate was subjected to a high velocity spin at 125,000 × g for 30 min at 4 °C. Supernatants were removed, and 25 μl were taken as a sample for SDS-PAGE. The pellets were re-extracted by vigorous shaking with 100 μl of lysis buffer and then centrifuged again at 125,000 × g for 30 min at 4 °C. The supernatants were discarded. Samples were analyzed by SDS-PAGE and immunoblot.

To assess the aggregation of newly imported mtHsp70s, radiolabeled Ssc1 was imported into isolated mitochondria. The import reaction was stopped after 15 min by the addition of valinomycin. Mitochondria were reisolated, washed once with SEM buffer, and subjected to the aggregation assay as described above. Samples were analyzed by SDS-PAGE and digital autoradiography.

**Miscellaneous Methods**—To determine enzymatic activity of aconitate in the mitochondrial matrix, isolated mitochondria were lysed and treated as described (30). Aconitate activity was

---

**TABLE 1**

Yeast strains used in this study

| Strain   | Genotype                  | Origin       |
|----------|---------------------------|--------------|
| YPH499   | MATa, ura3–52, lys2–801,  | EUROSCARF    |
|          | ade2–101, trpl1–Δ63,      |              |
|          | his3–Δ200, leu2–Δ1,       |              |
|          | ura3–Δ0, lys2–Δ0          |              |
| BY 4742  | MATa, his3–Δ1, leu2–Δ0,  | EUROSCARF    |
|          | lys2–Δ0, ura3–Δ0          |              |
| zim17–2  | (BG-Fomp3–20–2)          | Ref. 5       |
|          | MATa, ade2–101, his3–Δ200,|              |
|          | ura3–52, trpl1–Δ63,      |              |
|          | lys2–801, zim17–3:ADE2    |              |
|          | pfLzim17-Ts2-CEN          |              |
| zim17–3  | (BG-Fomp3-C1)            | Ref. 5       |
|          | MATa, ade2–101, his3–Δ200,|              |
|          | ura3–Δ200, leu2–Δ1,      |              |
|          | trpl1–Δ63, lys2–801,     |              |
|          | zim17–3a:LEU2,           |              |
| ssc1–2   | MATa, ade2–101, lys2–52, | Ref. 8       |
|          | leu2–3,112, trpl1,       |              |
|          | ssc1–2:LEU2              |              |
| ILY08    | MATa, ura3–52, lys2–801,  | This study    |
|          | ade2–101, trpl1–Δ63,     |              |
|          | his3–Δ200, leu2–Δ2,      |              |
|          | ZIM17:LEU2, respiritory  |              |
|          | incompetent               |              |
| ILY09    | MATa, ura3–52, lys2–801,  | This study    |
|          | ade2–101, trpl1–Δ63,     |              |
|          | his3–Δ200, leu2–Δ1,      |              |
|          | zim17–3a:LEU2,           |              |
|          | respiritory incompetent   |              |
| ILY10    | MATa, ura3–52, lys2–801,  | This study    |
|          | ade2–101, trpl1–Δ63,     |              |
|          | his3–Δ200, leu2–Δ1,      |              |
|          | zim17–3b:LEU2,           |              |
|          | respiritory incompetent   |              |
| ILY20    | MATa/a, leu2–Δ2, leu2–Δ2,| This study    |
|          | lys2–801/lys2–Δ0,        |              |
|          | URA3/ura3–Δ3, ade2–101/ADE2, trpl1–Δ63/ TRP1, his3–Δ200/HIS3, ZIM17:LEU2/ZIM17 | |
| ILY21    | MATa/a, leu2–Δ2, leu2–Δ2,| This study    |
|          | lys2–801/lys2–Δ0,        |              |
|          | URA3/ura3–Δ3, ade2–101/ADE2, trpl1–Δ63/ TRP1, his3–Δ200/HIS3, zim17–3a:LEU2/ZIM17 | |
| ILY22    | MATa/a, leu2–Δ2, leu2–Δ2,| This study    |
|          | lys2–801/lys2–Δ0,        |              |
|          | URA3/ura3–Δ3, ade2–101/ADE2, trpl1–Δ63/ TRP1, his3–Δ200/HIS3, zim17–3b:LEU2/ZIM17 | |
| ILY24    | MATa, trpl1–Δ63,         | This study    |
|          | LEU2, ZIM17:LEU2,        |              |
|          | respiratory competent    |              |
| ILY27    | MATa, trpl1–Δ63,         | This study    |
|          | LEU2, ZIM17:LEU2,        |              |
|          | respiratory competent    |              |
| ILY28    | MATa, trpl1–Δ63,         | This study    |
|          | LEU2, ZIM17:LEU2,        |              |
|          | respiratory competent    |              |
Zim17 Conditional Mutants Directly Affect mtHsp70 Functions

FIGURE 1. Generation and growth phenotype of temperature-sensitive zim17 mutants. A, surface representation of the Zim17 structure. The locations of the amino acid changes in the zim17-3a and zim17-3b mutants are indicated in red. B, magnification of the molecular environment of the amino acids Asp-111 and Asn-79 in the three-dimensional structure of Zim17 (Protein Data Bank code 2E2Z (20)). Asn-79 and Asp-111 are indicated in green, and neighboring residues and the four cysteines binding the zinc atom (sphere) are indicated in purple. Structures were visualized using the program Swiss-PdbViewer. C, growth phenotype of respiratory competent zim17-ts mutant yeasts. Serial dilutions of cells were spotted onto solid growth media, containing either glucose (YPD) or glycerol (YPG) as carbon source, and incubated at the indicated temperatures. D, loss of respiratory competence of zim17-ts mutants grown on a fermentable carbon source. Respiratory competent zim17-ts mutant cells were grown in liquid precultures in YPG at 25 °C, transferred to YPD, and further incubated at 25 °C for 48 h. At the indicated time points, samples were taken, and equal amounts of cells were plated on YPD and YPG solid media. After incubation at 25 °C, cfu were counted, and the ratio of cfu on YPG compared with YPD was determined. E, zim17-ts mutant strains were grown in YPG at 25 °C and subjected to non-permissive temperature (37 °C). At the indicated time points, samples were taken, plated on solid YPG, and incubated at 25 °C. Cfu were counted, and the viability in relation to zim17-wt cells was determined. Cells were kept at the logarithmic growth phase (OD 0.6–1.0) throughout the time courses of the experiment. Values shown are means ± S.E. (error bars) of four independent experiments.

RESULTS

Generation of Two Novel Temperature-sensitive Mutants of Zim17—To study the direct, short term consequences of a Zim17 loss of function on the cellular and on the mitochondrial level, we decided to screen for novel conditional mutant forms of the ZIM17 gene. A set of plasmids carrying zim17 mutations generated by error-prone PCR (26) was transformed into yeast cells lacking ZIM17 (zim17Δ) and screened for a temperature-sensitive growth phenotype. The mutant strain that showed the most severe temperature-sensitive phenotype (referred to as zim17-3) was selected for further characterization. Sequence analysis of zim17-3 revealed a mutation at position 111, where the aspartic acid in the highly conserved DHL motif was replaced by a glycine (D111G). An additional mutation was found at the less conserved residue 79, where asparagine in the direct neighborhood of the cysteines forming the zinc finger (Fig. 1, A and B) was replaced with a serine (N79S). However, even under the control of its own promoter, the expression of the zim17-3 mutant from a plasmid in the zim17Δ background resulted in a strong overexpression compared with WT protein levels. Accordingly, most of the mutant Zim17-3 was found as protein aggregate in the mitochondrial matrix (data not shown).

In order to avoid problems caused by the plasmid-borne copy, we decided to generate a genomically integrated form of the zim17-3 mutant. Because position Asp-111 was previously implicated in the interaction with Hsp70 chaperones (20), we decided to create a second mutant construct carrying only the mutation at position Asp-111 (Fig. 1A). The resulting mutants zim17-3a (D111G/N79S) and zim17-3b (D111G) as well as the WT control were integrated into the genome of the S. cerevisiae strain YPH499 according to published procedures (32, 33).

Genomic zim17 Mutants Show a Temperature-sensitive Phenotype Combined with Unstable Respiratory Competence—The cells that contained the integrated copies of the zim17 mutants showed a temperature-sensitive growth phenotype and were unable to grow at non-fermentable carbon sources (data not shown). To investigate whether the mutation in the ZIM17 gene was directly responsible for the respiratory deficient phenotype and the temperature-sensitive growth behavior, the genomic integrants were backcrossed against the WT strain BY4742 and subjected to a random spore analysis. As antici-
pated, all spores grew normally at 25 °C, whereas spores carrying the chromosomal zim17 mutations were unable to grow at an elevated temperature of 37 °C. When subjected to a non-fermentable carbon source, a subset of spores exhibited a respiratory competent phenotype, revealing that the loss of respiration is not a direct consequence of the loss of Zim17 function. However, even at a permissive temperature of 25 °C, we still observed that a significant ratio of clones derived from both mutant integrants (37% of zim17-3a and 75% of zim17-3b) were not able to grow on non-fermentable medium and therefore displayed a tendency to lose their respiratory competence. We tested the growth behavior of the zim17 mutant cells derived from respiratory competent spores in more detail. When non-fermentable conditions were maintained (YPD medium), both zim17 mutants showed a WT-like growth at 25 °C. At 37 °C, their growth was diminished in comparison with WT cells (Fig. 1C), confirming the initial temperature-sensitive growth phenotype. In contrast, when transferred to a growth medium containing the fermentable carbon source glucose (YPD medium), both zim17-3a and zim17-3b cells exhibited a petite phenotype at permissive temperature and were completely inviable at 37 °C (Fig. 1C).

Because both mutants showed a more severe phenotype on YPD, we assessed the stability of the respiratory competent phenotype by measuring how fast the mutant cells lost their ability to grow on YPG after a defined time on a fermentable medium. Respiratory competent zim17 mutant cells were grown in liquid YPD cultures at permissive temperature (25 °C), collected after different time points, and plated on both fermentable and non-fermentable media. Colony-forming units (cfu) on both media were counted, and the number of cfu on fermentable medium was set to 100%. The percentage of colonies that remained respiratory competent was determined. In contrast to the corresponding WT, both zim17-ts mutants rapidly lost their ability to respire even under permissive conditions, with less than 20% cells still able to grow on a non-fermentable carbon source after 48 h (Fig. 1D). To further assess the effects of elevated temperature on the viability of the respiratory-competent zim17 mutants, cells were grown at 37 °C on non-fermentable medium for up to 48 h and tested for survival rates on different growth media at permissive temperature by the cfu assay (see above). Compared with their corresponding WT strain, the viability of both zim17-3a and zim17-3b cells began to decrease after incubation for 12 h and reached levels of 52% (zim17-3a) and 35% (zim17-3b) of the corresponding WT cells after prolonged exposure to high temperature (Fig. 1E). In contrast to the shift to fermentable carbon sources, the shift to an elevated temperature did not induce a loss of respiratory competence. After exposure to 37 °C, the number of zim17 mutant cells that grew on YPG was indistinguishable from those grown on YPD (data not shown). Thus, incubation at non-permissive temperature did not induce respiratory deficiency as long as the mutant cells were kept under conditions where they are forced to respire.

Taken together, these data support the conclusion that the loss of respiration is not a direct effect of the loss of functional Zim17 but derives from secondary effects that are most likely due to the concomitant loss of functional mtHsp70s. Furthermore, the temperature-dependent growth defect of the zim17 mutant cells has to emanate from other, more specific effects of the zim17 mutation. For the further functional analysis of the zim17 mutant strains, only cells that originated from the initially respiratory competent spores were used. In our experiments, we distinguished between these respiratory competent mutant cells grown on YPG (zim17 rho+ ) and respiratory incompetent cells due to their growth on YPD (zim17 rho− ). Additionally, a 16-h in vivo heat shock was chosen to induce the ts phenotype, where only minimal effects on the overall viability of the mutant cells were observed (Fig. 1E).

**zim17-ts Mutants Show a Reduced Interaction with mtHsp70**—Because it has been proposed that amino acid Asp-111 is necessary for the interaction of Zim17 with mtHsp70s (20), we analyzed the interaction properties of the mutants Zim17-3a and Zim17-3b with soluble Ssc1. We performed co-immunoprecipitation assays with in vitro imported versions of the mutant Zim17 proteins (Fig. 2A) to assay the interaction under true in organello conditions and to avoid a bias due to aggregated or misfolded Ssc1 caused by in vivo expression of the zim17 mutants. Radiolabeled Zim17-WT, -3a, and -3b precursor proteins were imported into yeast WT mitochondria for 20 min at 25 °C. After the import reaction was stopped, samples were further incubated at 25 °C to allow the imported proteins to fold to their native state. After ATP depletion, mitochondria were lysed under native conditions, and co-immunoprecipitations with antibodies directed against Ssc1 were performed. To exclude the possibility that the signals were derived from an import-related interaction, we also imported Mdh1 (malate-dehydrogenase), a standard matrix-destined precursor that does not bind to Ssc1 after the import and folding have been completed. About 2% of the imported Zim17-WT was co-precipitated with Ssc1, whereas only 1.3% of Zim17-3a and 1.6% of Zim17-3b mutants compared with less than 0.1% of Mdh1 were found in the chaperone precipitates. Thus, although the association of the mutant Zim17 proteins with Ssc1 was compromised, it was not completely abolished.

**zim17-ts Mutants Show a Partial mtHsp70 Aggregation at Elevated Temperatures**—The two yeast mitochondrial Hsp70 proteins Ssc1 and Ssq1 are known to aggregate in mitochondria isolated from respiratory deficient zim17Δ cells grown at 25 °C (22). Hence, mitochondria isolated from zim17 rho+ and zim17 rho− cells were tested for mtHsp70 aggregation by high velocity centrifugation. The soluble matrix protein Sod2 was used as a control. In mitochondria isolated from zim17 rho− cells, more than 50% of the total amounts of Ssc1 and between 70 and 100% of the total amounts of Ssq1 could be found in the aggregate pellet fraction (Fig. 2B). This strong decrease of Hsp70 solubility is a direct effect of the zim17 mutations but not due to a loss of respiration because mitochondria from the comparable WT strain YPH499 lacking mitochondrial DNA (rho−) did not show any aggregation. In contrast, in zim17 rho+ mitochondria, the aggregation of both Ssc1 and Ssq1 and was dependent on a high temperature treatment (Fig. 2C). Although no aggregation was observed after growth at 25 °C, 30% of Scc1 and 70% of Ssq1 could be found in the aggregate pellet of zim17 rho+ 3a mitochondria obtained from cells grown 16 h at 37 °C.
aggregation was less severe under the same conditions (13% of total Ssc1 and 6% of total Ssq1). These values indicate a much less pronounced Hsp70 aggregation phenotype in our conditional zim17-3b mutants where a loss of respiratory competence is prevented. We did not observe a significant aggregation of other mitochondrial proteins in the zim17-ts mutants (data not shown), indicating that the observed aggregation propensities of mtHsp70 chaperones are related to their intrinsic biochemical properties.

We also tested the aggregation behavior of newly imported Ssc1 molecules in our zim17-ts mutants (Fig. 2C). Radiolabeled Ssc1 precursor proteins were imported into the matrix of isolated zim17rho-+ts and -WT mitochondria and tested for aggregation after the import reaction was completed. In contrast to the endogenous protein, only less than 1% of the newly imported Ssc1 aggregated in the zim17rho-+ts mutants at both permissive and non-permissive temperatures, indicating that the aggregation phenotype is not a problem of the general Hsp70 biogenesis process in zim17-ts mutants.

**zim17-ts Mutants Display Altered Amounts in the Relative Abundance of mtHsp70s and Their Co-chaperones**—We investigated the biochemical mechanisms underlying the maintenance of the respiratory competence of the zim17 mutants under non-fermentable conditions. Despite the genomic integration of the mutant genes, the proteins Zim17-3a and Zim17-3b exhibited slightly differing levels in comparison with WT mitochondria (Fig. 3A). In respiratory deficient zim17rho- cells, both mutant Zim17 proteins were overexpressed, whereas in respiratory competent zim17rho+ cells, the expression levels of the mutant proteins were lower. We therefore decided to take a closer look at the relative abundance of key proteins of the Ssc1 and Ssq1 chaperone systems in zim17 mutant mitochondria. Equal amounts of mitochondria from zim17rho-+ WT, -3a, and -3b cells grown at permissive and non-permissive temperatures were analyzed for the abundance of selected proteins by Western blot (Fig. 3B). We detected only a very slight reduction in the steady-state levels of the main mtHsp70 protein Ssc1 in zim17rho-+3a and zim17rho-+3b cells. After a 16-h incubation at 37 °C, Ssc1 showed a heat-stress-dependent up-regulation that was stronger in the mutants than in the WT cells. With regard to Mdj1, the J-protein of the Ssc1 system, its function during preprotein import, remained constant under all examined conditions.

In comparison with Ssc1, the levels of the second mtHsp70 protein, Ssq1, showed a significant reduction in zim17rho-+3a and -3b mitochondria both at permissive and non-permissive temperature. The levels of Jac1, the J-protein of the Ssq1 system, and Isu1, a scaffold protein that interacts with Ssq1 as a substrate during the synthesis of Fe/S-cluster proteins, remained constant in mitochondria of zim17rho-+WT, -3a, and -3b cells grown at permissive temperature. However, when cells were subjected to the heat treatment, the amounts of both Jac1 and Isu1 were strongly increased in comparison with WT cells (Fig. 3B). In contrast to non-fermentable conditions, no reduction of Mdj1 or Ssq1 levels were observed in zim17rho- mitochondria (Fig. 3C). The only prominent exception was a strong increase in the levels of Isu1, indicative of a defective mitochondrial Fe/S assembly system as a consequence of a loss of function of Zim17. Taken together, the relative up-regulation of the members of both matrix Hsp70 chaperone systems indicated the presence of adaptive processes taking place in zim17rho-+.
mutant cells, probably contributing to the maintenance of respiratory competence.

The Loss of Functional Zim17 Leads to Severe Defects in the Biogenesis of Fe/S Proteins—The Ssq1 chaperone system has been shown to play an important role in the biogenesis of Fe/S proteins in yeast (18, 34). We therefore analyzed the direct effects of zim17-ts mutations on this process. Because a shift to fermentable conditions resulted in a loss of respiratory competence of zim17 mutant cells over time, we first examined the levels of the citrate cycle Fe/S enzyme aconitase in zim17 mutants subjected to YPD (Fig. 4A). Indeed, after a 12-h incubation on fermentable medium, the levels of aconitase were reduced to non-detectable amounts in zim17 mutant cells grown on YPD (Fig. 4B). Similar results were observed in zim17rho- mutants grown on YPG (data not shown), the question remained if it still exhibited full enzymatic activity. Aconitase activity was normal in zim17rho- mutant cells grown on YPD; however, the activity in heat-treated zim17rho-3b cells obtained from cells grown at permissive temperature (Fig. 4C). Heat-treated yeast zim17rho-3a mitochondria displayed a strongly reduced aconitase activity (44% relative to the corresponding WT cells), whereas the activity in heat-treated zim17rho-3b mitochondria was similar to WT levels. In both cases, aconitase activities closely mirrored the different Ssq1 aggregation levels in the zim17 mutant strains.

We next analyzed whether the diminution of aconitase activity is due to an impaired iron-sulfur cluster assembly. Zim17 rho- cells were radiolabeled with $^{55}$Fe followed by immunoprecipitation of aconitase (31). The amount of $^{55}$Fe associated with immunoprecipitated aconitase represents a measure for the efficiency of iron-sulfur cluster formation. The de novo $^{55}$Fe incorporation into aconitase was diminished in zim17rho-3a cells yet only slightly decreased in the zim17rho-3b mutant (Fig. 4D). These decreases were comparable in extent with the loss in enzyme activity. Based on these observations, we conclude that the loss of aconitase was largely due to the defect in iron-sulfur cluster incorporation, most likely caused by a reduction of Ssq1 activity. In turn, the iron-sulfur protein defects in zim17rho- mutant mitochondria most probably explain the acquisition of a respiratory deficiency after long term exposure to fermentable conditions. However, the heat-induced defective phenotype of the respiratory
Zim17 Conditional Mutants Directly Affect mtHsp70 Functions

FIGURE 4. Effects of zim17-ts mutants on the biogenesis of Fe/S cluster-containing proteins. A, reduction of aconitase (Aco1) protein levels in respiratory competent zim17rho+ mutants after growth on non-fermentable medium. zim17-ts mutant cells grown in YPG at 25 °C were transferred to YPD medium as described in Fig. 2B. Whole cell yeast extracts taken at the indicated times were analyzed by SDS-PAGE. Western blot, and immunodecoration with antisera against the indicated proteins (Tom40, translocase of the outer membrane component of 40 kDa). B, loss of enzymes of the citric acid cycle and respiratory chain in zim17-ts mutants. Isolated mitochondria of the indicated yeast strains grown at permissive temperature on YPD (zim17rho+) and YPG (zim17rho-) were analyzed by Western blot and decoration with antisera against the indicated proteins (Cit1, citrate synthase; Rip1, Rieske iron-sulfur protein; CoxIV, subunit 4 of the cytochrome c oxidase). C, aconitase activity in mitochondria isolated from zim17rho+ mutant cells grown on YPG at permissive and non-permissive temperature. D, 56Fe incorporation efficiencies into Aco1 in zim17rho+ mutant cells grown at 30 °C. Values shown are means ± S.E. (error bars) of at least three independent experiments.

The zim17-3a Mutant Exhibits a Defect in the Import of Matrix-targeted Preproteins—Because Ssc1 represents the most important mtHsp70, we examined the effects of a loss of Zim17 function on the activity of Ssc1. The essential function of Ssc1 is closely correlated with its important role in the import of preproteins into the mitochondrial matrix via the TIM23 complex. Therefore, we analyzed the import phenotype of respiratory competent zim17rho+ -ts mutants grown at permissive temperature or at 37 °C for 16 h. We used the well characterized model preprotein Su9(86)-DHFR, which consists of the N-terminal presequence and the first 20 residues of the mature part of subunit 9 of the Fo-ATPase from Neurospora crassa fused to mouse DHFR, for in organello import experiments. The radio-labeled precursor protein was imported into isolated mitochondria, and the amounts of processed and protease-protected protein were quantified. zim17rho+ -3a mitochondria obtained from heat-treated cells showed a strong reduction of import efficiency, whereas the import of zim17rho+ -3b mitochondria was indistinguishable from WT (Fig. 5A). Mitochondria from cells grown at permissive temperature did not show any import deficiencies (data not shown). Conversely, the import of an inner membrane protein, the ADP/ATP translocator (AAC), which is imported into mitochondria via an Ssc1-independent pathway, was not affected by the zim17-3a mutation (Fig. 5B). In order to exclude the possibility that defects in the respiratory chain were the cause for the defect in the import of matrix-destined preproteins, we measured the electrochemical gradient across the inner mitochondrial membrane (data not shown). No alterations of the membrane potential were observed in zim17rho+ -3a and zim17rho+ -3b mitochondria, confirming that the import deficiency is a primary consequence of the zim17-3a mutation and correlates directly with its loss of function.

zim17-ts Mutants Display a Stable Interaction of Ssc1 with ATP and the Preprotein Translocase—As zim17-3a mitochondria showed only a partial (30%) aggregation of the main import-related Hsp70 chaperone Ssc1, we reasoned that the severe import defect was caused by a more direct functional defect of the chaperone system. To this end, we examined the behavior of Ssc1 at different points of its enzymatic cycle that are relevant for the import reaction. Ssc1 is recruited to the translocase via the scaffold protein Tim44. At this position, it is able to exert an inwardly directed translocation force (“pulling”) on the incoming protein that is driven by a conformational change induced by ATP hydrolysis. We tested the ability of Ssc1 to generate an import-driving force on preproteins in transit by a specific assay utilizing the matrix-targeted preprotein b2(167)Δ-DHFR, consisting of the presequence and the first 89 N-terminal residues of the mature cytochrome b2 fused to the entire mouse DHFR molecule. The addition of the DHFR ligand MTX stabilizes the folding state of the DHFR domain and arrests b2(167)Δ-DHFR in the import channel, whereas the aminoterminal segment of the preprotein can reach the matrix compartment, thereby interacting with the chaperone machinery. In the case of an active translocation force, the MTX-stabilized DHFR domain is pulled tightly to the surface of the outer mitochondrial membrane and remains largely resistant to externally added proteases (13). The amounts of processed, protease-resistant b2(167)Δ-DHFR were determined and normalized to the total amount of imported protein that was accumulated in the import channel. We observed no difference in the amounts of protease-resistant translocation intermediates between WT and zim17rho+ mutant mitochondria, implying
that the interaction of Ssc1 with the translocase complex of the inner mitochondrial membrane was still intact (Fig. 5C). For control purposes, we used the mutant ssc1-2, a temperature-sensitive variant of Ssc1 that carries a mutation in the substrate-binding domain and is known to exhibit a reduced inwardly directed translocation force (13).
Zim17 Conditional Mutants Directly Affect mtHsp70 Functions

Because Zim17 is reported to bind to the ATPase domain of Ssc1 preferably under nucleotide-free conditions and thereby most likely promotes its folding into a native state (35), we assessed the effect of the zim17-3a mutant on the ability of Ssc1 to bind ATP. Mitochondria from heat-treated and non-heat-treated zim17rho° cells were lysed under native conditions and applied to ATP-agarose columns (Fig. 5E). Ssc1 from the mutant mitochondria exhibited ATP binding efficiencies similar to Ssc1 from WT mitochondria under both conditions, showing that the general ability of Ssc1 to bind ATP is not influenced by the Zim17 mutations. Because the import activity of Ssc1 is closely correlated with a functional inner membrane preprotein translocase complex (TIM23), we performed pull-down experiments with Protein A-tagged Tim23 that was expressed in zim17-WT and -3a cells from a plasmid. Consistent with the results above, no differences were observed in the amounts of co-purified Ssc1 and translocase subunits, confirming that the TIM23 import motor is intact in mutant mitochondria (Fig. 5G).

The zim17-3a Mutant Shows an Inadequate Substrate Interaction of Ssc1 during the Import of Preproteins to the Mitochondrial Matrix—We next asked if the zim17-3a mutant would be able to influence the interaction of Ssc1 with newly imported substrate proteins in organelle. For this purpose, we performed import experiments with radiolabeled Su9(86)-DHFR followed by co-immunoprecipitations with antibodies against Ssc1 (Fig. 5H). The values of the co-precipitated preproteins were normalized against the amount of soluble Ssc1 to avoid a bias due to protein aggregation. About 5% of newly imported Su9(86)-DHFR at permissive temperature and about 10% at non-permissive temperature could be co-precipitated with Ssc1 from zim17-WT mitochondria, values that are in the typical range for preproteins interacting with Ssc1 at the import site (13). As expected, in contrast, a significant decrease in the amount of co-precipitated Su9(86)-DHFR was observed in zim17rho°-3a mitochondria already under permissive conditions. After a 16-h heat treatment at 37 °C, only about half of the amount found in WT mitochondria was recovered with Ssc1 (Fig. 5H). Moreover, the interaction with substrate in the mutant was prolonged. Although the interaction with substrate was transient and reduced ~2-fold after 15 min of postimport incubation in the wild type, substrates in heat-treated zim17rho°-3a mitochondria stayed attached to Ssc1. Taken together, the zim17rho°-3a mutant showed a diminished interaction of Ssc1 with incoming preproteins at the translocation channel that potentially contributed to the import deficiency observed in the mutant.

The Interaction of Ssc1 with Unfolded Substrates in the Mitochondrial Matrix Is Not Affected in the zim17-3a Mutant—Because the substrate interaction of Ssc1 can be divided into two phases, one import-related (assisted by Pam18) and the other related to folding processes in the matrix (assisted by Mdj1), we asked if the second type of interaction was also affected by Zim17 mutations. We performed an in vitro substrate interaction assay using the model chaperone substrate RCMLA, an intrinsically unfolded protein and efficient artificial chaperone substrate (Fig. 5F). Mitochondrial soluble protein extracts were prepared and subjected to a high speed centrifugation step to exclude aggregated Ssc1 from the reaction. The soluble fractions were incubated with immobilized RCMLA, and bound Ssc1 was eluted with excess ATP. Surprisingly, the binding efficiency of Ssc1 to RCMLA was unchanged in the zim17rho°-3a mutant under permissive and non-permissive conditions (Fig. 5F). We finally assessed the folding capacity of Ssc1 in the zim17rho°-3a mutant by determining the amounts of protease-resistant folded DHFR after import of radiolabeled Su9(86)-DHFR into mitochondria. After the import, reaction mitochondria were lysed and treated with proteinase K. The amounts of protease-resistant DHFR were determined relative to the overall amount of imported protein (Fig. 5D). As expected, ssc1-2 mutant mitochondria that were used as a control displayed a strong folding defect. In contrast, we did not detect any significant folding defects in the zim17rho°-3a mutant relative to WT mitochondria. In summary, our results show an import-specific decrease in the substrate binding of Ssc1 in zim17-3a mutant suggesting a direct role of Zim17 during the import-related Hsp70 substrate interaction assisted by class III J-proteins (in this case by Pam18).

DISCUSSION

Based on a comprehensive biochemical and cell biological characterization of novel conditional yeast mutants, we propose that the protein Zim17 exerts a direct influence on the cellular functions of its cognate Hsp70 chaperones in the mitochondrial matrix. Previous experiments using yeast deletion mutants (zim17Δ) showed that both mitochondrial Hsp70s, Ssc1 and Ssq1, had a high tendency to aggregate and that the ATPase domain of newly imported Ssc1 is unable to fold properly in the absence of Zim17 (35, 36). However, a direct analysis of Zim17 function under these conditions was difficult due to the accumulation of severe secondary defects caused by the aggregation of mtHsp70s. Our analysis of a previously published conditional (temperature-sensitive) mutant strain, zim17-2 (26), exhibited that the mutant protein was virtually undetectable in isolated mitochondria (data not shown). This behavior is most likely due to the presence of an additional mutation in its presequence that interfered with its mitochondrial import, whereas the overall stability of the mutant protein did not seem to be affected (data not shown). Thus, concerning its biochemical behavior, the zim17-2 strain rather corresponded to a full deletion strain. We therefore identified a new conditional mutant, zim17-3a, from a mutant library generated by random mutagenesis that exhibited a severe phenotype. The Zim17-3a protein contained two amino acid exchanges (N79S and D111G). The aspartic acid at position 111 belongs to the conserved DNL motif of Zim17 and is believed to be part of the interface for the interaction with Ssc1 (20). Similarly, the human Zim17 orthologue DNLI/HEP showed a dependence on residue Asp-111 in its interaction with the corresponding Hsp70 chaperone HSP9A (21). In order to distinguish the effects of the different point mutations, we also constructed the strain zim17-3b, which only contained the D111G mutation. Accordingly, both mutant proteins displayed a decreased yet not completely abolished binding to mtHsp70s in the mitochondrial matrix. Hence, our independent identification of an amino acid exchange at position Asp-111 generated by random
Zim17 Conditional Mutants Directly Affect mtHsp70 Functions

Mutagenesis indicates the significance of this site for the functional integrity of Zim17.

The conditional mutants were integrated into the yeast genome to replace the WT gene. A backcross of the integrated zim17 mutations against WT yeasts allowed us to generate zim17 mutants cells with a respiratory competent phenotype (zim17Δ) as long as they were forced to grow under non-fermentable conditions. Growth of zim17Δ cells on non-fermentable medium was strongly impeded by higher temperature as expected. Ssq1, the Fe/S biogenesis chaperone, was strongly down-regulated and showed an almost complete aggregation in the zim17Δ-3a mutant. This behavior was reflected by significant alterations in the amounts of the Ssq1 substrate protein Isu1 and its J-domain partner Jac1 after the 37 °C heat treatment. The Isu1 up-regulation and the presence of Jac1 are critical for the survival of yeast in the absence of Ssq1 or other components of the mitochondrial Fe/S cluster assembly machinery (37). Because the levels of the main mtHsp70 Ssc1 were also increased, we conclude that growth of the mutant cells under non-fermentable conditions induces an adaptive process in the gene expression, leading to an improved protein import defect at elevated temperatures in the zim17Δ-3a mutant. This behavior was reflected by significant alterations in the amounts of the Ssq1 substrate protein Isu1 and its J-domain partner Jac1 after the 37 °C heat treatment. The Isu1 up-regulation and the presence of Jac1 are critical for the survival of yeast in the absence of Ssq1 or other components of the mitochondrial Fe/S cluster assembly machinery (37). Because the levels of the main mtHsp70 Ssc1 were also increased, we conclude that growth of the mutant cells under non-fermentable conditions induces an adaptive process in the gene expression, leading to an improved conservation of mitochondrial functions, in particular respiratory competence. Although we observed no significant reductions in the levels of respiratory enzymes or Fe/S proteins in respiratory competent zim17Δ-3a mitochondria, we measured a loss of activity of the Fe/S protein aconitate going along with a defect in the de novo incorporation of its Fe/S cluster. Because the levels of Ssq1 are reduced in the zim17Δ-3a mutants, it is likely that the role of Ssq1 is partially taken over by Ssc1 under these conditions. This is corroborated by the observation that an artificial overexpression of Jac1 could shift the function of Ssq1 in Fe/S cluster biogenesis to Ssc1 and rescue the growth defects of ssq1Δ mutants (13).

In contrast, when kept on a fermentable carbon source like glucose, both yeast mtHsp70s aggregated already at a permissive temperature of 25 °C, and the mutant cells (zim17Δ) rapidly lost their respiratory competence. Under these conditions, we observed a complete loss of iron-sulfur cluster-containing proteins but also of other enzymes of the respiratory chain and the citrate cycle. No adaptive responses of the chaperone systems were observed except for a strong up-regulation of the Fe/S biogenesis enzyme Isu1. Because a loss of metabolic enzymes and a subsequent inability to respire was also observed in S. cerevisiae cells with defects in the Fe/S cluster biogenesis (17, 38, 39), we conclude that the cumulative phenotypic defects were mainly caused by a defect in Fe/S cluster biogenesis.

Hence, the temperature-sensitive lethal phenotype of our zim17Δ-Δ mutants most likely correlates with a functional defect of the main mtHsp70 Ssc1. Indeed, because the essential function of Ssc1 is its role in the import of matrix-destined precursor proteins, we observed a strong preprotein import defect at elevated temperatures in zim17Δ-3a mitochondria. We considered that a possible reason for the defective import was caused by the observed aggregation of Ssc1. However, because only 30% of Ssc1 were aggregating in the zim17Δ-3a mutants, it is not likely that the strong import deficiency would be solely caused by the diminished protein levels of Ssc1. Furthermore, the TIM23 translocase complex was essentially unaltered, and the steady state pulling activity of Ssc1 on preproteins in transit was indistinguishable in zim17Δ-3a mutant and WT mitochondria. Because we also did not observe any defect of Ssc1 to interact with nucleotides in our zim17 mutants, its import motor functions in general did not seem to be affected as such.

Nevertheless, co-immunoprecipitation experiments revealed a reduced interaction of Ssc1 with newly imported proteins and in particular a less efficient release of the substrate polypeptide after the import reaction in the zim17Δ-3a mutant. In contrast, the interaction of Ssc1 with general Hsp70 substrates in vitro (RCMLA) was not altered. Thus, the substrate interaction deficiency seemed to be restricted to import-related processes that are mediated by Ssc1. The two polypeptide binding processes of Ssc1, during the import reaction at the inner membrane and during folding reactions in the matrix, can be distinguished by the requirement for different J-domain co-chaperones. During folding reactions in the matrix compartment, Ssc1 functionally interacts with the class I J-protein Mdj1. In contrast, during preprotein import, Ssc1 interacts with the class III J-protein Pam18. The second mtHsp70 chaperone Ssc1 interacts with Jac1, another class III J-protein. Due to its zinc finger domain, it has been presumed earlier that Zim17 might act as one polypeptide part of a “fractured” J-protein, providing a substrate-binding motif for Type III J-proteins like Pam18 and Jac1 (22). Our observations of a direct effect of Zim17 on the import-related substrate binding activity of Ssc1 support an aspect of the fractured J-protein hypothesis. However, because Jac1 has retained some intrinsic substrate binding ability (19) and Ssc1 bound at the import channel is in close proximity to incoming substrate proteins, it is unlikely that Zim17 directly delivers substrate polypeptides to the respective Hsp70 (7). Instead, we hypothesize that Zim17 rather regulates the substrate affinity of mtHsp70s. The up-regulation of the specific Ssq1 co-chaperone Jac1 in the zim17Δ-Δ mutants as a potential reaction to the diminished substrate affinity of Ssq1 would support this conclusion. In support of the “fractured” J-protein hypothesis, DNLZ/HEP has been shown to enhance the ATPase activity of mtHsp70 (40), a feature that has not been observed in yeast Zim17 so far. We presume that DNLZ/HEP may have gained this feature to further support the stable binding of substrates to mtHsp70. This, together with our results, would favor a model in which Zim17 binding to Ssc1 occurs during or after the initial binding of substrate and stabilizes the Hsp70-substrate complex after ATP hydrolysis to allow a more efficient interaction. A similar task has been suggested for zinc center II in the C-terminal substrate-binding domain of DnaJ. Whereas zinc center I seems to be directly involved in the interaction with unfolded substrates, it has been proposed that zinc center II provides a second site of interaction with the Hsp70 chaperone and contributes to the high affinity interaction of Hsp70s with substrate proteins (41). In accordance with these findings, the zim17Δ-3a mutant shows a generally more severe phenotype than the zim17Δ-3b mutant, probably due to its second mutation at position 79, which is in close proximity to the zinc-chelating residues of Zim17. Correlating with a J-like activity of Zim17, it must also be noted that the proposed binding site of Zim17 near the Hsp70 domain interface has also been implicated in the interaction with J-proteins in general.
Our data do not exclude the possibility that Zim17 is also able to interact with the ATPase domain of nucleotide-free, newly imported mtHsp70s or stays attached to intermediates in the enzymatic cycle to prevent their misfolding and subsequent aggregation. However, we were not able to detect newly imported Ssc1 in the aggregate pellets of zim17Δ mutant cells under both permissive and non-permissive conditions, excluding an aggregation-protective role of Zim17 during mtHsp70 biogenesis. In our model, mtHsp70 aggregation in the zim17 mutant mitochondria could be caused by an accumulation of substrate-free intermediates of the enzymatic cycle due to a reduced substrate affinity. It seems to be reasonable to assume that an enhancement of substrate affinity and the prevention of aggregation through the binding of Zim17 might just compensate a reduced substrate affinity. It seems to be reasonable to assume that an enhancement of substrate affinity and the prevention of aggregation through the binding of Zim17 might just be a reduced substrate affinity. It seems to be reasonable to assume that an enhancement of substrate affinity and the prevention of aggregation through the binding of Zim17 might just be a reduced substrate affinity. It seems to be reasonable to assume that an enhancement of substrate affinity and the prevention of aggregation through the binding of Zim17 might just be a reduced substrate affinity. It seems to be reasonable to assume that an enhancement of substrate affinity and the prevention of aggregation through the binding of Zim17 might just be a reduced substrate affinity. It seems to be reasonable to assume that an enhancement of substrate affinity and the prevention of aggregation through the binding of Zim17 might just be a reduced substrate affinity.

Taken together, this work provides new insights into direct functional effects of the yeast protein Zim17 on mitochondrial Hsp70 chaperone activities, in particular during the import of nucleus-encoded preproteins and the biogenesis of Fe/S proteins. Our findings point to a novel supportive role of Zim17 in enhancing the substrate affinity of their cognate Hsp70 chaperones in cooperation with class III J-proteins that lack the cysteine-rich substrate binding domains of the typical class I J-proteins.

Acknowledgments—We thank U. Gerken for excellent technical assistance and Dr. G. Cenini and C. Rüb for helpful discussion and for critically reading the manuscript.

REFERENCES

1. Frydman, J. (2001) Folding of newly translated proteins in vivo. The role of molecular chaperones. Annu. Rev. Biochem. 70, 603–647
2. Voos, W., and Röttgers, K. (2002) Molecular chaperones as essential mediators of mitochondrial biogenesis. Biochim. Biophys. Acta 1592, 51–62
3. Mayer, M., and Bukau, B. (2005) Hsp70 chaperones. Cellular functions and molecular mechanism. Cell Mol. Life Sci. 62, 670–684
4. Young, J. C., Agashe, V. R., Siegers, K., and Hartl, F. U. (2004) Pathways of chaperone-mediated protein folding in the cytosol. Nat. Rev. Mol. Cell Biol. 5, 781–791
5. Hartl, F. U., Bracher, A., and Hayer-Hartl, M. (2011) Molecular chaperones in protein folding and proteinostasis. Nature 475, 324–332
6. Vogel, M., Mayer, M. P., and Bukau, B. (2006) Allosteric regulation of mitochondrial Hsp70 chaperones involves a conserved interdomain linker. J. Biol. Chem. 281, 38705–38711
7. Kampinga, H. H., and Craig, E. A. (2010) The HSP70 chaperone machin-ery. J proteins as drivers of functional specificity. Nat. Rev. Mol. Cell Biol. 11, 579–592
8. Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C., and Zylcic, M. (1991) Escherichia coli DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. Proc. Natl. Acad. Sci. U.S.A. 88, 2874–2878
9. Kelley, W. L. (1999) Molecular chaperones. How J domains turn on Hsp70s. Curr. Biol. 9, R305–R308
10. Walsh, P., Bursa, D., Law, Y. C., Cyr, D., and Lighthow, T. (2004) The J-protein family. Modulating protein assembly, disassembly and translocation. EMBO Rep. 5, 567–571
11. Neupert, W., and Herrmann, J. M. (2007) Translocation of proteins into mitochondria. Annu. Rev. Biochem. 76, 723–749
12. Schmidt, O., Pfanner, N., and Meisinger, C. (2010) Mitochondrial protein import. From proteomics to functional mechanisms. Nat. Rev. Mol. Cell Biol. 11, 655–667
13. Voisine, C., Craig, E. A., Zufall, N., von Ahsen, O., Pfanner, N., and Voos, W. (1999) The protein import motor of mitochondria. Unfolding and trapping of preproteins are distinct and separable functions of matrix Hsp70. Cell 97, 565–574
14. Truscott, K. N., Voos, W., Frazier, A. E., Lind, M., Li, Y., Geissler, A., Dudek, J., Müller, H., Sickmann, A., Meyer, H. E., Meisinger, C., Guiard, B., Rehling, P., and Pfanner, N. (2003) A J-protein is an essential subunit of the presequence translocate-associated protein import motor of mitochondria. J. Cell Biol. 163, 707–713
15. Rowley, N., Prip-Buus, C., Westermann, B., Brown, C., Schwarz, E., Barrell, B., and Neupert, W. (1994) Mdj1p, a novel chaperone of the DnaJ family, is involved in mitochondrial biogenesis and protein folding. Cell 77, 249–259
16. Westermann, B., Gaume, B., Herrmann, J. M. (1996) Role of the mitochondrial DnaJ homolog Mdj1p as a chaperone for mitochondrial synthesized and imported proteins. Mol. Cell. Biol. 16, 7063–7071
17. Lill, R., Hoffmann, B., Molk, S., Pierik, A. J., Rietzschel, N., Stehling, O., Utarska, M. A., Webert, H., Wilbrecht, C., and Mühlenhoff, U. (2012) The role of mitochondrial chaperone iron-sulfur protein biogenesis and iron metabolism. Biochim. Biophys. Acta 1823, 1491–1508
18. Dukkwievicz, R., Marszalek, J., Schilke, B., Craig, E. A., Lill, R., and Mühlenhoff, U. (2006) The Hsp70 chaperone Ssq1p is dispensable for iron-sulfur cluster formation on the scaffold protein Isu1p. J. Biol. Chem. 281, 7801–7808
19. Ciesielski, S. J., Schilke, B. A., Osiupik, J., Bigelow, L., Mulligan, R., Majewski, J., Joachimiak, A., Marszalek, J., Craig, E. A., and Dukkwievicz, R. (2012) Interaction of J-protein co-chaperone Jac1 with Fe-S scaffold Isu is dispensable in vivo and conserved in evolution. J. Mol. Biol. 417, 1–12
20. Momose, T., Ohshima, C., Maeda, M., and Endo, T. (2007) Structural basis of functional cooperation of Tim15/Zim17 with yeast mitochondrial Hsp70. EMBO Rep. 8, 664–670
21. Zhai, P., Yu, M. T., Hoff, K. G., and Silberg, J. I. (2011) A conserved histidine in human DNLR/HEP is required for stimulation of HSPA9 ATPase activity. Biochem. Biophys. Res. Commun. 408, 589–594
22. Burri, L., Vascotto, K., Feddersen, S., Tiedt, B., Hall, M. N., and Lithgow, T. (2004) Zim17, a novel zinc finger protein essential for protein import into mitochondria. J. Biol. Chem. 279, 50243–50249
23. Sichting, M., Mokranjac, D., Azem, A., Neupert, W., and Hell, K. (2005) Maintenance of structure and function of mitochondrial Hsp70 chaperones requires the chaperone Hcp1. EMBO J. 24, 1046–1056
24. Willmund, F., Hinnenberger, M., Nick, S., Schulz-Raaffelt, M., Mühlhaus, T., and Schrodta, M. (2008) Assistance for a chaperone. Chlamydomonas HEP2 activates plastidic HSP70B for co-chaperone binding. J. Biol. Chem. 283, 16363–16373
25. Yamamoto, H., Momose, T., Yatsukawa, Y., Ohshima, C., Ishikawa, D., Sato, T., Tamura, Y., Ohwa, Y., and Endo, T. (2005) Identification of a novel member of yeast mitochondrial Hsp70-associated motor and chaperone proteins that facilitates protein translocation across the inner membrane. FEBS Lett. 579, 507–511
26. Sanjuan Sklarz, L. K., Guiard, B., Rissler, M., Wiedemann, N., Kozjak, V., van der Laan, M., Lohaus, C., Marcus, K., Meyer, H. E., Chacinska, J., Joachimiak, A., Marszalek, J., Craig, E. A., and Dutkiewicz, R. (2005) Inactivation of the mitochondrial heat shock protein zim17 leads to aggregation of matrix Hsp70s followed by pleiotropic effects on morphology and protein biogenesis. J. Mol. Biol. 351, 206–218
27. Ryan, M. T., Voos, W., and Pfanner, N. (2001) Assaying protein import into mitochondria. Methods Cell Biol. 65, 189–215
28. Becker, D., Krayl, M., Strub, A., Li, Y., Mayer, M. P., and Voos, W. (2009) Impaired interdomain communication in mitochondrial Hsp70 results in the loss of inward-directed translocation force. J. Biol. Chem. 284, 2934–2946
29. Böd, B., Grimminger, V., and Walter, S. (2005) Substrate binding to the molecular chaperone Hsp104 and its regulation by nucleotides. J. Biol. Chem. 280, 38170–38176
30. Bender, T., Lewenz, I., Franken, S., Baitzel, C., and Voos, W. (2011) Mitochondrial enzymes are protected from stress-induced aggregation by
mitochondrial chaperones and the Pim1/LON protease. *Mol. Biol. Cell* **22**, 541–554

31. Pierik, A. J., Netz, D. J., and Lill, R. (2009) Analysis of iron-sulfur protein maturation in eukaryotes. *Nat. Protoc.* **4**, 753–766

32. Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F., and Cullin, C. (1993) A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **21**, 3329–3330

33. Lorenz, M. C., Muir, R. S., Lim, E., McElver, J., Weber, S. C., and Heitman, J. (1995) Gene disruption with PCR products in *Saccharomyces cerevisiae*. *Gene* **158**, 113–117

34. Dutkiewicz, R., Schilke, B., Knieszner, H., Walter, W., Craig, E. A., and Marszalek, J. (2003) Ssq1, a mitochondrial Hsp70 involved in iron-sulfur (Fe/S) center biogenesis. Similarities to and differences from its bacterial counterpart. *J. Biol. Chem.* **278**, 29719–29727

35. Blamowska, M., Sichting, M., Mapa, K., Mokranjac, D., Neupert, W., and Hell, K. (2010) ATPase domain and interdomain linker play a key role in aggregation of mitochondrial Hsp70 chaperone. *J. Biol. Chem.* **285**, 4423–4431

36. Blamowska, M., Neupert, W., and Hell, K. (2012) Biogenesis of the mitochondrial Hsp70 chaperone. *J. Cell Biol.* **199**, 125–135

37. Andrew, A. J., Dutkiewicz, R., Knieszner, H., Craig, E. A., and Marszalek, J. (2006) Characterization of the interaction between the J-protein Jac1p and the scaffold for Fe-S cluster biogenesis, Isu1p. *J. Biol. Chem.* **281**, 14580–14587

38. Andrew, A. J., Song, J.-Y., Schilke, B., and Craig, E. A. (2008) Posttranslational regulation of the scaffold for Fe-S cluster biogenesis, Isu. *Mol. Biol. Cell* **19**, 5259–5266

39. Hausmann, A., Samans, B., Lill, R., and Mühlenhoff, U. (2008) Cellular and mitochondrial remodeling upon defects in iron-sulfur protein biogenesis. *J. Biol. Chem.* **283**, 8318–8330

40. Zhai, P., Stanworth, C., Liu, S., and Silberg, J. J. (2008) The human escort protein Hep binds to the ATPase domain of mitochondrial hsp70 and regulates ATP hydrolysis. *J. Biol. Chem.* **283**, 26098–26106

41. Linke, K., Wolfram, T., Bussemer, J., and Jakob, U. (2003) The roles of the two zinc binding sites in DnaJ. *J. Biol. Chem.* **278**, 44457–44466