Jac1, a mitochondrial J-type chaperone, is involved in the biogenesis of Fe/S clusters in *Saccharomyces cerevisiae*

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A minor Hsp70 chaperone of the mitochondrial matrix of *Saccharomyces cerevisiae*, Ssq1, is involved in the formation or repair of Fe/S clusters and/or mitochondrial iron metabolism. Here, we report evidence that Jac1, a J-type chaperone of the mitochondrial matrix, is the partner of Ssq1 in this process. Reduced activity of Jac1 results in a decrease in activity of Fe/S containing mitochondrial proteins and an accumulation of iron in mitochondria. Fe/S enzyme activities remain low in both *jac1* and *ssq1* mutant mitochondria even if normal mitochondrial iron levels are maintained. Therefore, the low activities observed are not solely due to oxidative damage caused by excess iron. Rather, these molecular chaperones likely play a direct role in the normal assembly process of Fe/S clusters.

molecular chaperones | Hsp70 | Hsp40 | DnaJ | mitochondria

The Fe/S proteins participate in numerous cellular processes in virtually all organisms. Recent studies indicate that a conserved system exists to facilitate the assembly and insertion of Fe/S clusters into proteins (1–3). The idea that cellular machinery facilitates the process of Fe/S cluster assembly comes from studies of nitrogen-fixing microorganisms such as *Azotobacter vinelandii*. In such organisms, the Fe and MoFe proteins comprising the enzyme nitrogenase make up 5–10% of total cellular protein (for review, see ref. 4). A specialized system appears to have evolved for the mobilization of Fe and S for assembly into the Fe/S clusters of nitrogenase. In particular, the *nifS* and *nifU* gene products are thought to function specifically in the acquisition of sulfur and iron, respectively.

Proteins related to NifS, NifU, and other Nif proteins also are found in non-nitrogen-fixing bacteria and eukaryotes, suggesting that these proteins may serve a broader role in assembly of Fe/S clusters in proteins. *Escherichia coli* IscS, which is related to *nifS*, is thought to use its cysteine desulfurase activity to donate sulfur for the assembly of Fe/S clusters (5, 6). Consistent with this idea, an *E. coli* strain lacking IscS has reduced specific activity of a number of Fe/S proteins (7, 8).

In *Saccharomyces cerevisiae*, these related gene products are located in mitochondria. Mutations in genes related to *nifS* and *nifU* (*NFS1*, *NFU1*, and *ISU1/2*) result in lowered activity of Fe/S-containing enzymes (9–12). In addition, strains having reduced amounts of mitochondrial ferredoxin or Isa (encoded by *YAH1* and *ISA1/2*, respectively) have lowered activity of Fe/S-containing proteins (13–15). Reduction of the activity of all of these mitochondrial proteins not only leads to a decrease in the activity of Fe/S-containing enzymes but also an increase in mitochondrial iron levels, suggesting a role for all of these proteins in a common process.

In *E. coli*, several of the genes having sequence similarity to the *nif* genes are in a gene cluster called *isc* (iron sulfur cluster) (6). The genes *hscA* and *hscB*, encoding an Hsp70 and a J-type molecular chaperone, respectively, reside within this cluster, raising the idea that molecular chaperones may be involved in the assembly or repair of Fe/S clusters. In fact, a minor Hsp70 of the yeast mitochondrial matrix, Ssq1, has been linked to iron metabolism and assembly/repair of Fe/S clusters. In addition to growing extremely poorly at temperatures that are optimal for growth of wild-type yeast and below, Δ*ssq1* cells have reduced activity of mitochondrial Fe/S proteins and accumulate10-fold higher than normal levels of iron in their mitochondria (9, 16, 17). Genetic results also support a connection between the process of Fe/S cluster assembly and Ssq1 function. A Δ*ssq1* mutation is synthetically lethal with a Δ*nfu1* mutation (9).

Hsp70s work together with J-type chaperones (also referred to as Hsp40s or DnaJs) as part of a "chaperone machine" (18). For example, the *E. coli* Hsp70 DnaK and the Hsp40 DnaJ function in refolding of denatured proteins *in vivo* (19, 20). J-type chaperones interact with Hsp70s via the conserved J domain to stimulate the ATPase activity of Hsp70s, thus stabilizing the binding of unfolded polypeptide substrates. A genetic screen revealed a candidate for a J-type partner for Ssq1, Jac1. In addition to *ssq1* and *nfs1* mutants, a *jac1* mutant was identified as a suppressor of metabolic defects associated with the absence of the copper/zinc superoxide dismutase (Sod1). *JAC1*, an essential gene, encodes a protein containing a putative J domain, the signature domain of J-type chaperones, immediately adjacent to an NH_2-terminal segment characteristic of proteins imported into mitochondria (10).

To determine whether Jac1 and Ssq1 function as partners, we set out to further characterize this J-type chaperone and its relationship to iron metabolism. Indeed, Jac1 is a protein of the mitochondrial matrix. Under semipermissive conditions, a *JAC1* mutant strain accumulates iron in its mitochondria and has reduced activity of Fe/S-containing enzymes. Both *ssq1* and *jac1* mutant cells grown under conditions such that iron mitochondrial levels are normal still have lowered activity of Fe/S enzymes, suggesting that these chaperones are directly involved in the process of assembly of Fe/S clusters.

Materials and Methods

Yeast Strains, Plasmids, Media, and Chemicals. *JAC1* was obtained by PCR-amplifying genomic DNA from position −350 to +824 using Pfu1 polymerase (Stratagene) and cloned into the pRS series of vectors (21). To obtain a null allele of *JAC1*, DNA from +93 to +517 was replaced with the *ADE2* gene. A heterozygous *jac1* diploid of *PJ53* (22) carrying *JAC1* on a plasmid was sporulated. Δ*jac1* haploids carrying *JAC1* on a plasmid were

Abbreviations: SDH, succinate dehydrogenase; HPD, histidine, proline, aspartic acid tripeptide.

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isolated by dissecting onto YP media (1% yeast extract, 2% peptone) containing 3% glycerol as a carbon source to ensure maintenance of respiratory competency.

Mutants of *JAC1* were constructed by site-directed PCR mutagenesis and cloned into pRS313. In the case of the *JAC1* mutant $jac1_{\Delta32}$, a chromosomal integrant was constructed. This integrant had the same phenotype as a strain expressing $Jac1_{\Delta32}$ from a plasmid (data not shown). An expression vector for *in vitro* transcription/translation of *JAC1* was made by creating a *Ecl*136II site 12 bp upstream of the translation start site and introducing this fragment into pGEM-7zf+ (Promega). To construct an expression vector for purification of the Jac1 protein, six histidine codons were introduced at the 3′ end of the *JAC1* protein coding region by PCR. The PCR product containing the entire *JAC1* coding sequence tagged with six histidine codons was cloned under the control of a galactose-inducible promoter using the pYES2.0 vector (Promega). This construct, pYES2$JAC1_{His}$, rescued the inviability of *JAC1* null cells when grown on media containing galactose (data not shown).

ssq1, isu1, and *nfu1* strains have been described (9, 16). *BJ3497* (23), which is defective in proteinase A, was used for purification of Jac1. Unless otherwise indicated, yeast were grown in YP media with 2% glucose, 2% galactose, or 3% glycerol and 2% ethanol as the carbon source. Low iron media (0.67% yeast nitrogen base without iron, copper, and amino acids; Bio101, Carlsbad, CA) were supplemented with amino acids, 1 μM copper sulfate, and a carbon source. Complete synthetic media lacking histidine or containing 5-fluoroorotic acid were prepared as described (24). All chemicals, unless stated otherwise, were purchased from Sigma.

Translocation of Jac1 into and Fractionation of Mitochondria. Methods for isolation of mitochondria and import experiments have been described (25). For submitochondrial localization, 50 μg of mitochondrial protein isolated from wild-type cells was fractionated as described (16). Samples then were analyzed by immunoblot analysis using polyclonal antibodies against Jac1 (this study), $Cytb_2$, or Mge1 (26) using the Renaissance detection kit from New England Nuclear.

Respiratory Enzyme, Mitochondrial Iron, and Cytochrome Measurements. Activities of the respiratory enzymes were measured in isolated mitochondria. Succinate dehydrogenase (SDH)-$cytbc_1$ activity was measured by using succinate as the substrate as described (27), except 2,6-dichloroindophenol was used in place of cytochrome *c* (9). Aconitase activity was measured by monitoring the decrease in the absorbance of the substrate isocitrate at 240 nm as described (12, 28). Mitochondrial iron levels were determined colorimetrically with ferene as described (9, 29). Data were normalized to the protein content of the mitochondrial samples. The spectrophotometric analysis of the cytochromes was performed in whole cells after immersion of the cuvette in liquid nitrogen as reported (30). Protein determinations were performed by using the Bio-Rad protein assay with ovalbumin as a standard.

$jac1_{\Delta32}$ cells were grown at 30°C and then shifted to 37°C or 38.5°C and incubated overnight before harvest, using either glycerol/ethanol or galactose as a carbon source and harvested at an A_{600} of 0.1–0.4 or 0.5–1.0, respectively. In the case of $\Delta ssq1$ experiments, strains were grown at 34°C to an A_{600} of 0.5–1.0 in galactose-based medium.

Protein Purification and N-Terminal Sequencing. $Jac1_{His}$ was purified from mitochondria isolated by affinity chromatography using a Ni^{2+} column. Fractions were concentrated by trichloroacetic acid precipitation and separated on SDS/PAGE gel and transferred onto Sequi-Blot polyvinylidene difluoride membrane (Bio-Rad). The bands containing $Jac1_{His}$ protein were excised, and the N-terminal sequence was obtained by using Edman degradation methods by the Protein and Nucleic Acid Chemistry Laboratories at Washington University School of Medicine, St. Louis. Mdj1 was purified as described (31).

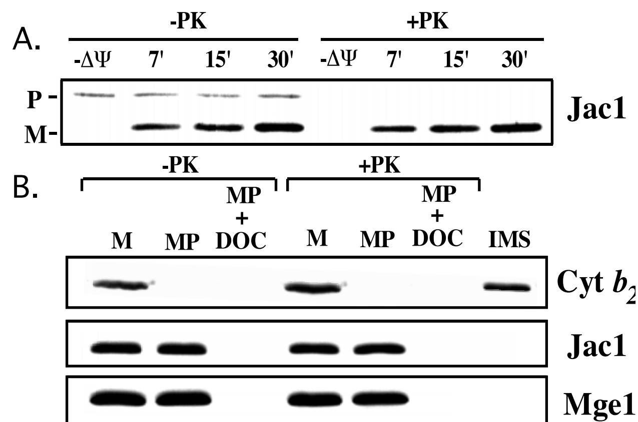

Fig. 1. Jac1 of the mitochondrial matrix. (*A*) Translocation of radiolabeled Jac1 into isolated mitochondria was carried out in the absence ($-\Delta\Psi$) or presence of a membrane potential for 7, 15, and 30 min. PK, proteinase K; p, precursor; m, mature. (*B*) Mitochondria (M) were separated into mitoplasts (MP) and intermembrane space (IMS) fractions. An equivalent portion of mitoplasts was disrupted by addition of deoxycholate detergent (MP + DOC). Equivalent amounts of the fractions were either treated (+PK) or not treated (−PK) with proteinase K. Immunoblot analysis was carried out with antibodies specific for Jac1, cytochrome b_2 (Cyt b_2) as a marker for the intermembrane space, or the matrix protein Mge1 after separation by electrophoresis.

Immunoblot Analysis. Immunoblot analysis was carried out as described (32). For generation of an Mdj1 antibody, an in-frame fusion between glutathione *S*-transferase and the entire mature form of Mdj1 was constructed in pGEX-KT (33). The fusion protein was expressed in *E. coli* and purified by adsorption to glutathione-agarose beads. To generate rabbit antisera for Jac1, an N-terminal His-tagged version of Jac1 was expressed in *E. coli* and purified by adsorption to Ni^{2+} beads.

Results

Jac1 Is Localized to the Mitochondrial Matrix. As noted by Strain *et al.* (10), the putative protein product of the *JAC1* gene contains an N-terminal extension relative to other J-type chaperones that have characteristics of typical mitochondrial presequences. Therefore, we tested whether Jac1 is a mitochondrial protein. To determine whether Jac1 can be imported into mitochondria *in vitro*, a radiolabeled protein was synthesized in rabbit reticulocyte lysate and added to energized mitochondria. Jac1 was efficiently translocated into isolated mitochondria in a reaction dependent on a membrane potential, indicative of translocation across the inner mitochondrial membrane. In the process, Jac1 underwent cleavage to a lower molecular weight form (Fig. 1*A*).

To directly determine the normal cellular location of Jac1, purified mitochondria were subjected to hypotonic treatment to disrupt the outer membrane. Jac1 remained within the mitoplasts, whereas cytochrome b_2, a protein of the intermembrane space, was released (Fig. 1*B*). Jac1, like the matrix protein Mge1, became susceptible to proteinase K only after disruption of the inner membrane with detergent. Together, these results indicate that Jac1 is a protein of the mitochondrial matrix.

To determine the NH_2 terminus of the mature form of Jac1, we constructed a version of *JAC1* containing a polyhistidine tag at the C terminus to facilitate purification of the protein. This tagged version of Jac1 rescues the inviability of a $\Delta jac1$ strain (data not shown). Tagged Jac1 protein purified from isolated

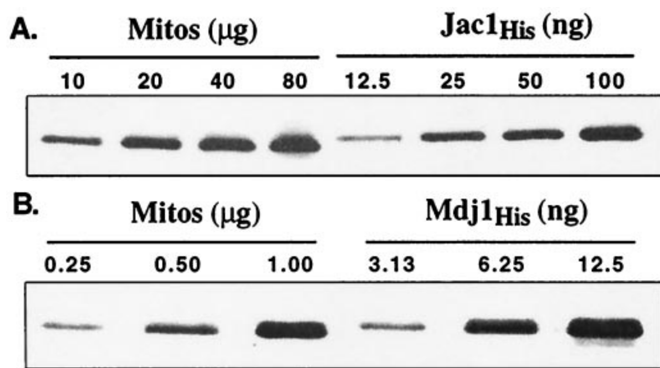

Fig. 2. Relative levels of Jac1 and Mdj1 in mitochondria. Immunoblot band intensities of predetermined concentrations of purified His-tagged Jac1 or Mdj1 were compared with those from mitochondrial proteins isolated from wild-type yeast cells. The indicated amounts of mitochondrial proteins and purified His-tagged Jac1 (*A*) or Mdj1 (*B*) were separated by electrophoresis and subjected to immunoblot analysis using polyclonal antibodies specific for Jac1 (*A*) or Mdj1 (*B*).

mitochondria was found to have the sequence TSTFYEL at the NH_2 terminus, indicating that the precursor form has a 10-aa extension that is removed upon translocation into mitochondria.

To determine the amount of Jac1 in mitochondria, varying amounts of mitochondrial lysates were separated by SDS/PAGE next to varying amounts of purified His-tagged Jac1 (Fig. 2*A*). Immunoblot analysis was performed by using Jac1-specific antibodies. By comparison of the signal from isolated mitochondria with the purified Jac1 proteins, we calculated that Jac1 makes up about 0.15% of mitochondrial protein. For comparison, we performed a similar analysis of Mdj1, another Hsp40 of the mitochondrial matrix. Mdj1 makes up about 1.1% of mitochondrial protein (Fig. 2*B*).

The J Domain of Jac1 Is Important for Its Function. The essential Jac1 protein has been classified as a J-type chaperone because of the presence of a J-domain found in other chaperones of this class (10). Because *JAC1* is an essential gene, we began our analysis of the importance of the J domain with a previously isolated *JAC1* mutation, a deletion of a single codon encoding for the aspartic acid at position 32 within putative helix II of the J domain ($jac1_{\Delta 32}$) (10). $jac1_{\Delta 32}$ cells that have maintained mitochondrial DNA grew as well as wild-type cells on a variety of carbon sources between 23°C and 34°C, including glucose and glycerol (Fig. 3*A*; data not shown). The previous report (10) that $jac1_{\Delta 32}$ cells were unable to grow on nonfermentable carbon sources might have been due to the lack of wild-type mitochondrial DNA in the isolates tested. The mutant is slightly temperature-sensitive, however, as growth at 37°C on nonfermentable carbon sources is slow compared with wild-type cells. To determine the relative level of mutant Jac1 and wild-type protein, we carried out immunoblot analysis of mitochondrial extracts from the two strains. As can be seen in Fig. 3*B*, mitochondria harboring the mutant protein contain only about one-fifth the amount of Jac1 protein as wild-type mitochondria. Therefore, it is likely that the slight temperature-sensitive growth defect observed is due to the reduced amount, rather than a functional defect, of the mutant protein. However, because $jac1_{\Delta 32}$ cells grow so well at 30°C, far less Jac1 is needed for robust growth under optimal growth conditions than is normally present.

To determine whether the J domain of Jac1 is important for its function, we constructed another *JAC1* mutant gene having alterations in the J domain. Because the hallmark of a J domain is a highly conserved tripeptide, histidine, proline, aspartic acid (HPD), we constructed a mutation that results in the replacement of the HPD with three alanines ($jac1_{A3}$). To test whether the $jac1_{A3}$ mutant could rescue the inviability of a *JAC1* deletion strain, a plasmid containing the $jac1_{A3}$ mutant gene was transformed into a strain carrying a deletion of *JAC1* on the chromosome and a wild-type copy of *JAC1* on a centromeric plasmid having a *URA3* gene. When the resulting strain was plated onto media containing 5-fluoroorotic acid, which selects for cells having lost the *URA3*-containing plasmid carrying the wild-type *JAC1* gene, viable colonies were recovered (Fig. 4*A*).

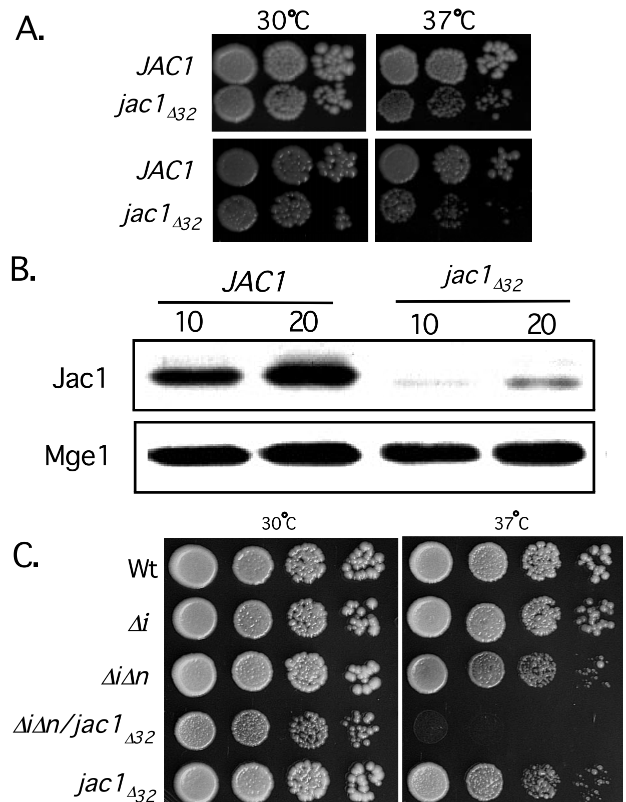

Fig. 3. Growth defects associated with $jac1_{\Delta 32}$. (*A*) 1:10 serial dilutions of strains deleted for *JAC1* and carrying plasmids expressing either wild-type *JAC1* or $jac1_{\Delta 32}$ were spotted onto YP plates containing glucose (*Upper*) or glycerol (*Lower*) and incubated at 30°C or 37°C for 4 days. (*B*) Mitochondrial proteins (10 or 20 μg) isolated from strains carrying either wild-type *JAC1* or $jac1_{\Delta 32}$ grown in YP glycerol/ethanol media at 30°C were separated by electrophoresis and subjected to immunoblot analysis with Jac1 (*Upper*) or Mge1 (*Lower*) specific antibodies. (*C*) Serial dilutions of the indicated strains were spotted on YP-glucose plates and incubated at 30°C or 37°C for 4 days. Δ*nfu1* (Δ*n*); Δ*isu1*(Δ*i*).

However, $jac1_{A3}$ cells grew very slowly at 23°C and 30°C and were inviable at 37°C on glucose-based media. On glycerol-based media, $jac1_{A3}$ growth was even less robust compared with wild-type cells, but viability on this carbon source indicated that the mutant cells had maintained mitochondrial DNA. To determine whether the severe phenotype of $jac1_{A3}$ was due to low expression of Jac1 or to the compromised function of the mutant protein, we assayed the expression of $Jac1_{A3}$. Because of the extreme growth defect, we tested $Jac1_{A3}$ levels in cells having both wild-type Jac1 tagged with a C-terminal polyhistidine tag and $Jac1_{A3}$. Because His-tagged Jac1 migrates more slowly than Jac1 lacking the tag, we were able to determine the expression level of the mutant Jac1 protein by immunoblot analysis (Fig. 4*B*). Similar levels of mutant and wild-type Jac1 were observed, indicating that this mutant protein is approximately as stable as wild-type protein. We conclude that the J domain of Jac1 is extremely important for its function. Whether the $jac1_{A3}$ muta-

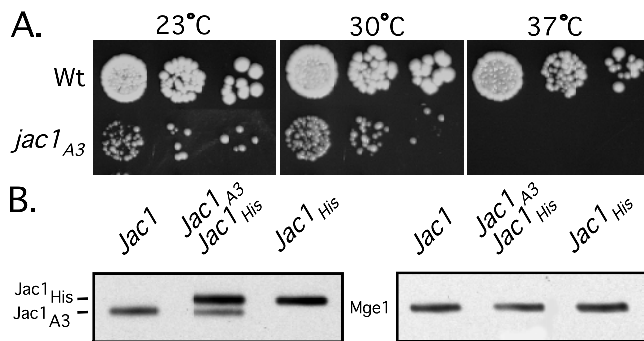

Fig. 4. Importance of the J domain HPD motif in Jac1 function. (*A*) 1:10 serial dilutions of each strain were spotted onto YP plates containing glucose and incubated at 30°C for 3 days, or at 23°C or 37°C for 4 days. (*B*) Mitochondria were prepared from strains expressing wild-type Jac1, Jac1 with a polyhistidine tag ($Jac1_{His}$), or both the tagged Jac1 and the HPD mutant protein ($Jac1_{A3}$ + $Jac1_{His}$). Extracts were prepared and proteins were separated by SDS/PAGE. After transfer, the immobilized proteins were subjected to immunoblot analysis using either Jac1-specific antibodies (*Left*) or antibodies specific for the mitochondrial matrix protein Mge1.

tion completely disrupts J domain function awaits biochemical analysis.

Genetic Interactions Among *JAC1*, *SSQ1*, *NFU1*, and *ISU* Genes. Because Jac1 is a candidate J-type partner for Ssq1, the mitochondrial Hsp70 implicated in the assembly of Fe/S clusters, we tested for interactions between Δ*ssq1* and $jac1_{\Delta32}$ mutations. We were unable to recover a Δ*ssq1* $jac1_{\Delta32}$ strain, consistent with the idea that Ssq1 and Jac1 function together.

We also examined genetic interactions between $jac1_{\Delta32}$ and deletions of *NFU1* and *ISU1*, which encode components involved in the assembly of Fe/S cluster, as *SSQ1* mutations have shown genetic interactions with mutations in these genes (9). Δ*nfu1* cells grow very robustly. Δ*isu1* cells have a temperature-sensitive growth defect on nonfermentable carbon sources, whereas Δ*isu1* Δ*nfu1* cells are more compromised for growth, being somewhat temperature-sensitive even on glucose-based media (ref. 9 and Fig. 3*C*). Comparison of the growth of strains carrying the single $jac1_{\Delta32}$, Δ*isu1*, or Δ*nfu1* mutations with those carrying the $jac1_{\Delta32}$ mutation in combination with either the Δ*isu1* or Δ*nfu1* mutations revealed little differences in growth. However, we noted a dramatic temperature-sensitive growth defect of $jac1_{\Delta32}$ Δ*isu1* Δ*nfu1* (Fig. 3*C*). Therefore, genetic interactions exist between a mutation in *JAC1* and mutations in genes encoding components of the Fe/S assembly machinery, Nfu1 and Isu1, as was found with a deletion of *SSQ1*. Together, these genetic results suggest that Ssq1 functions with Jac1 and that these chaperones function with the Fe/S center assembly machinery.

***JAC1* Mutant Cells Are Defective in Fe/S Enzyme Activities and Accumulate Iron in Their Mitochondria.** Because of these genetic interactions with genes known to be involved in Fe/S cluster formation and the involvement of the Hsp70 Ssq1 in this process, we tested the activity of two mitochondrial Fe/S-containing enzymes, aconitase and SDH, in mitochondria isolated from $jac1_{\Delta32}$ cells. Cells were grown in glycerol/ethanol-based media to ensure that mtDNA was maintained, as cells lacking mtDNA, and thus, unable to grow on nonfermentable carbon sources, have no SDH activity and lowered aconitase activity. Mitochondria were harvested from cells grown at 30°C but shifted to either 37°C or 38.5°C and incubated overnight before harvest (Fig. 5*A*). At 30°C, SDH activity in $jac1_{\Delta32}$ mitochondria was reduced only slightly, being about 80% of the activity found in wild-type mitochondria. However, mitochondria from $jac1_{\Delta32}$ cells harvested at 37°C and 38.5°C had approximately 60% and 30%, respectively, of the activity found in mitochondria isolated from wild-type cells treated in the same manner. Similar results were obtained when aconitase activity was assayed. At 30°C, aconitase activity was reduced by about 20% compared with wild type, but $jac1_{\Delta32}$ mitochondria from cells harvested at 37°C and 38.5°C had approximately 35% and 25% of wild-type activity, respectively. As a control, activity of NADH dehydrogenase, a mitochondrial protein lacking an Fe/S cluster, was monitored. The NADH dehydrogenase activity of wild-type mitochondria and $jac1_{\Delta32}$ mutant mitochondria were similar under all conditions described (data not shown). In summary, shift to the temperatures at which growth of $jac1_{\Delta32}$ is compromised resulted in a decrease in Fe/S protein activity.

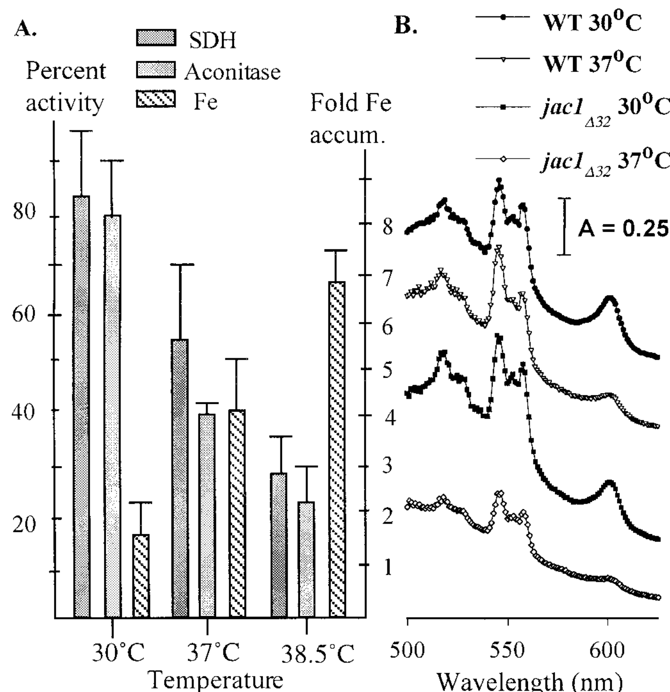

Fig. 5. Biochemical analysis of $jac1_{\Delta32}$ cells. Wild-type and $jac1_{\Delta32}$ cells were grown at 30°C and shifted to the indicated temperatures. (*A*) Enzyme activities and iron content of mitochondria were determined. At least triplicate samples were analyzed for at least three independent mitochondrial preparations Wild-type enzyme activity was set at 100% and iron levels at 1. Bars indicate. the average values, and standard deviations are indicated. (*B*) Low-temperature absorption spectra of wild-type and $jac1_{\Delta32}$ were measured. The absorption maxima for cytochromes are as follows: c, 547 nm; c1, 553 nm; b, 558 nm; a and a3, 600 nm.

Several mutants that have lowered activity of Fe/S proteins accumulate higher than normal levels of iron in their mitochondria (3). Therefore, we assayed the levels of iron in the mitochondria used for the enzyme assays described above. An increase in iron levels was observed (Fig. 5*A*). Whereas the level of iron was increased only 1.8-fold in $jac1_{\Delta32}$ mutant mitochondria from cells harvested at 30°C, mitochondria from cells harvested at 37°C and 38.5°C had 3.9-fold and 6.7-fold higher levels of iron than wild-type mitochondria, respectively. Therefore, in a strain having reduced Jac1 function, the activity of mitochondrial Fe/S proteins was reduced, and mitochondrial iron levels were increased. Furthermore, the level of iron was inversely correlated with the level of reduction in enzyme activity.

Iron is not only present in Fe-S centers in mitochondrial proteins but also in heme of cytochromes. To begin to test whether chaperones have a role in heme metabolism, we com-

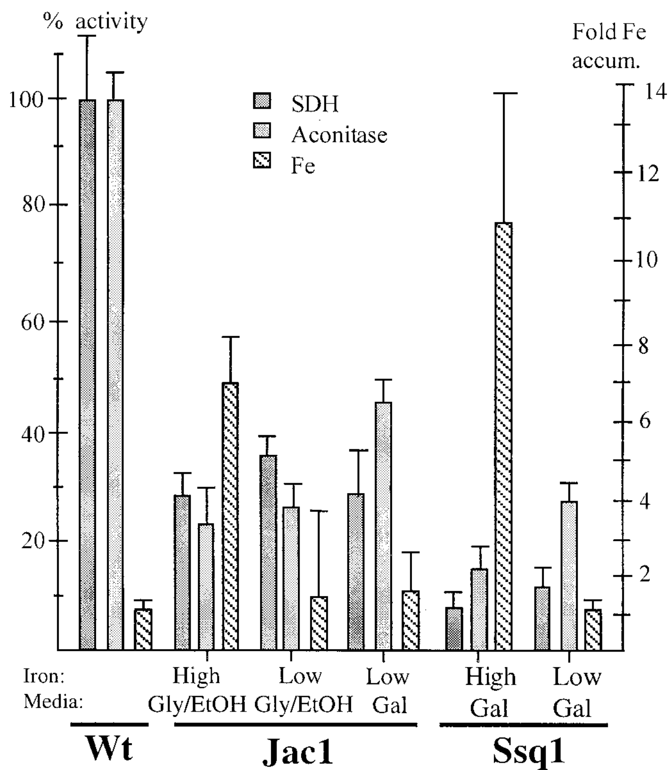

Fig. 6. Effects of reduced iron on Δ*ssq1* and $jac1_{\Delta 32}$ mitochondria. Mitochondria were isolated from wild-type, $jac1_{\Delta 32}$, and Δ*ssq1* cells grown in the indicated carbon sources in media containing high or low levels of iron. Enzyme activities and iron content were determined. At least triplicate samples were analyzed by using at least two preparations of mitochondria. Bars indicate the average values, and standard deviations are indicated.

pared the amount of cytochromes in wild-type and $jac1_{\Delta 32}$ cells at 30°C and after a shift to 37°C, making use of the unique spectral properties of different cytochromes. $jac1_{\Delta 32}$ cells showed a reduced level of all cytochromes at 37°C (Fig. 5*B*). Whether this reduction is a reflection of direct involvement in heme metabolism or down-regulation of cytochromes because of decreased activity of the Fe/S-containing components of the electron transport chain is unclear.

***JAC1* and *SSQ1* Mutants with Normal Mitochondrial Iron Levels Have Decreased Fe/S Protein Activities.** Increased levels of iron can result in the damage of Fe/S clusters because of the increased production of oxygen radicals. Therefore, the question arises as to whether the decrease in activity of Fe/S enzymes is a direct result of the decrease in Jac1 function or the indirect result of the increase in iron levels in mitochondria. To address this question, we grew cells in media containing low levels of iron, which had previously been shown to result in nearly normal levels of mitochondrial iron in two mutant strains that normally accumulate mitochondrial iron, *ssq1* and *nfs1* (12, 32). Mitochondria were isolated from cells grown at 30°C in glycerol/ethanol-based media, then shifted to 38.5°C 12–15 h before harvest. Under such conditions, the level of iron that accumulated in the mitochondria of $jac1_{\Delta 32}$ cells decreased from 6.7-fold higher than wild type to only 2.2-fold higher. However, the SDH and aconitase activities were still significantly lower than wild-type levels, 35% and 27% of wild-type activity, respectively (Fig. 6). Growth in galactose-based media resulted in even lower iron levels in the *JAC1* mutant mitochondria, only 1.6-fold higher than in wild-type mitochondria. mtDNA was maintained in $jac1_{\Delta 32}$ cells under these growth conditions (data not shown). However, SDH and aconitase activities remained low, 30% and 40% of wild-type activity, respectively (Fig. 6). The iron levels present in these mitochondria, isolated from cells shifted to the semipermissive temperature of 38.5°C, are even lower than that present in mitochondria isolated from the same strain grown at 30°C in iron-rich media, in which case enzyme activities are near wild-type levels. Therefore, the reduced activity of Fe/S enzymes found in the $jac1_{\Delta 32}$ mutant strain cannot be attributed to the increased iron levels in the mitochondria.

Previously, *ssq1* cells were shown to accumulate 10-fold more iron in their mitochondria, and the level of iron could be reduced to normal levels when grown in media containing low levels of iron. When grown in iron-rich media, the activity of both SDH and aconitase was reduced to 6% and 10% of wild-type levels in Δ*ssq1* mutant cells (9, 10). However, the SDH and aconitase activities were still significantly reduced, having 11% and 25% of wild-type level of activity, respectively, when Δ*ssq1* mitochondria were isolated from cells grown in media containing low levels of iron (Fig. 6). As was the case with $jac1_{\Delta 32}$ mitochondria, we conclude that most of the decrease in enzyme activities in mitochondria lacking Ssq1 are not an indirect effect of increased iron levels. Instead, we propose that both Jac1 and Ssq1 function are required for the assembly of Fe/S-containing proteins as an Hsp70/Hsp40 chaperone machine.

Discussion

The data reported here indicate that the Hsp40-related protein, Jac1, of the mitochondrial matrix functions in the pathway of assembly of Fe/S clusters. The involvement of a J-type chaperone in the assembly of Fe/S proteins may well be broadly conserved. The cluster of genes in *A. vinelandii*, *E. coli*, and *Haemophilus influenzae* involved in this process includes *hscB*, which encodes a J-type chaperone. Typically, J-type chaperones function with Hsp70s (34). For several reasons, Ssq1 is a promising candidate for the Hsp70 partner of Jac1. Like the $jac1_{\Delta 32}$ mutant, *ssq1* mutants show genetic interactions with genes encoding the machinery involved in Fe/S cluster formation. For example, an *nfu1* mutation was originally isolated based on its synthetic lethal interaction with Δ*ssq1,* and Δ*ssq1* mitochondria have low Fe/S enzyme activity (9, 10). In addition, *JAC1* and *SSQ1* mutants, as well as an *NFS1* mutant, were isolated as suppressors of an *SOD1* mutation, suggesting a connection among these genes (10).

However, differences between *SSQ1* and *JAC1* are evident. In the strain background analyzed here, Jac1 is essential, whereas lack of Ssq1 results in a cold-sensitive growth phenotype, suggesting the Ssq1 may not carry out an absolutely essential function. However, Ssc1, another Hsp70 present in the mitochondrial matrix at much higher levels than Ssq1, is able to partially substitute for Ssq1. Overproduction of *SSC1* partially suppresses not only the growth defect caused by the absence of Ssq1 but also the decrease in activity of Fe/S-containing enzymes (32). Therefore, the function carried out by Ssq1 may be essential. Jac1 and Ssq1 function may well be equally important for Fe/S center assembly.

A second J-type chaperone that has been implicated in the folding of newly imported proteins, Mdj1, also exists in the mitochondrial matrix (35). However, there is no indication that Mdj1 is capable of carrying out the functions of Jac1. Normally, Mdj1 is 7- to 8-fold more abundant than Jac1, but even overexpression of Mdj1 does not allow growth of Δ*jac1* cells, nor does overexpression of Jac1 suppress the growth phenotype of a Δ*mdj1* strain (data not shown). The basis of this functional difference between these two mitochondrial Hsp40s is unresolved. There may be functional differences in the J domains. The J domains of the cytosolic J-type chaperones Ydj1 and Sis1 can substitute for one another, as demonstrated by the functionality of chimeric genes containing exchanged J domains (36).

However, such chimeric constructs were not functional when substitutions of the J domains between Mdj1 and Jac1 were tested. Neither a Jac1 chimera containing the Mdj1 J domain nor a Mdj1 chimera containing a substituted Jac1 J domain could functionally replace either Jac1 or Mdj1 (data not shown). It is perhaps more likely that the ability of Jac1 to bind particular protein substrates is the basis of the functional difference. In support of this idea, it has recently been reported that the *E. coli* IscU, which is closely related to Isu1/2 of yeast, binds both HscB and HscA, the J-type molecular chaperone and Hsp70 encoded in the *isc* gene cluster, respectively (37, 38). As expected of a substrate of an Hsp70, IscU cooperates with HscB to stimulate the ATPase activity of HscA several hundred-fold (38, 39).

Interestingly, Jac1 is present at much higher concentrations than Ssq1 in the mitochondrial matrix. This relatively high concentration of a J-type chaperone relative to its partner Hsp70 is unusual. Most J-type chaperones are present in lesser amounts than their Hsp70 partner, consistent with their presumed catalytic role in stimulating ATPase activity and presenting substrate proteins (40). Perhaps Jac1 functions with Ssc1 at certain times. If so, it may compete with Mdj1 for interaction with Ssc1, necessitating a higher concentration in the matrix. However, these high levels of Jac1 are not essential for viability, as the mutant protein encoded by the temperature-sensitive $jac1_{\Delta 32}$ allele used in this study is present in about 5-fold lower amounts than wild-type protein without dramatic effects on growth, particularly under optimal growth conditions.

Like many yeast strains carrying mutations in genes involved in assembly/repair of Fe/S clusters, iron also accumulates in the mitochondria of the *JAC1* mutant strain we have analyzed. A fundamental question about the function of molecular chaperones in mitochondria is whether Ssq1 and Jac1 play a direct role in the assembly process. Alternatively, they could play a role in the regulation of iron metabolism. For example, the increase in iron resulting from the disruption of chaperone function could cause the destruction of Fe/S centers because of the increased production of destructive oxygen radicals. However, the persistent low Fe/S enzyme activity even when mutant cells have low mitochondrial iron levels points to a direct role in Fe/S cluster formation, one of assembly rather than repair of damaged clusters. The biochemical interactions of HscA, HscB, and IscU of *E. coli* also point to a role in Fe/S cluster assembly (38, 39), but the exact nature of this role remains to be resolved. These molecular chaperones could facilitate the folding or assembly of the components of the Fe/S cluster assembly machinery such as Isu1/2, Nfu1, and Nfs1. Alternatively, they could be more intimately involved in the insertion of the Fe/S cluster itself into recipient proteins. Regardless, specialized molecular chaperones seem to have evolved and been maintained throughout evolution as central players in the assembly of Fe/S clusters. It is also possible that Ssq1 and Jac1 play a role beyond Fe/S cluster assembly, as the level of cytochromes was decreased after shift of the *JAC1* mutant to high temperature. Whether this reduction is due to a direct role of chaperones in heme metabolism or a reflection of the coregulation of components of the electron transport chain remains to be resolved.

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