

# Mitochondrial enzymes are protected from stress-induced aggregation by mitochondrial chaperones and the Pim1/LON protease

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**ABSTRACT** Proteins in a natural environment are constantly challenged by stress conditions, causing their destabilization, unfolding, and, ultimately, aggregation. Protein aggregation has been associated with a wide variety of pathological conditions, especially neurodegenerative disorders, stressing the importance of adequate cellular protein quality control measures to counteract aggregate formation. To secure protein homeostasis, mitochondria contain an elaborate protein quality control system, consisting of chaperones and ATP-dependent proteases. To determine the effects of protein aggregation on the functional integrity of mitochondria, we set out to identify aggregation-prone endogenous mitochondrial proteins. We could show that major metabolic pathways in mitochondria were affected by the aggregation of key enzyme components, which were largely inactivated after heat stress. Furthermore, treatment with elevated levels of reactive oxygen species strongly influenced the aggregation behavior, in particular in combination with elevated temperatures. Using specific chaperone mutant strains, we showed a protective effect of the mitochondrial Hsp70 and Hsp60 chaperone systems. Moreover, accumulation of aggregated polypeptides was strongly decreased by the AAA-protease Pim1/LON. We therefore propose that the proteolytic breakdown of aggregation-prone polypeptides represents a major protective strategy to prevent the *in vivo* formation of aggregates in mitochondria.

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## INTRODUCTION

To perform their biological function, proteins have to adopt the correct three-dimensional structure. Whereas, under defined *in vitro* conditions, proteins are able to fold without any assistance, in the crowded environment of a living cell, the presence of molecular chaperones is mandatory for accurate initial folding (Hartl and Hayer-Hartl, 2009). Moreover, in a natural environment, the native structure of proteins is often challenged by stress conditions, such as elevated temperature or increased concentrations of reactive oxygen species (ROS). Partial or complete unfolding of a polypep-

ptide chain usually results in the exposure of internal hydrophobic stretches, thus raising the probability of nonnative contacts, leading to irregular protein interactions and ultimately aggregation. Apart from the mostly irreversible inactivation of cellular enzymes, the accumulation of aggregates itself represents a potentially toxic process (Brignull *et al.*, 2007). Therefore, cells have evolved an elaborate protein quality control network composed of molecular chaperones and proteases to secure protein homeostasis, even when the functional integrity of cellular proteins is challenged (Bukau *et al.*, 2006). Generally, in order to avoid protein aggregation, polypeptides are stabilized by chaperones, which may also promote their refolding to the functional state. Alternatively, cellular function may be secured by the specific proteolytic breakdown of aggregation-prone proteins in case all refolding efforts fail. The fact that formation of protein aggregates plays a prominent role in the pathogenesis of many human diseases, especially neurodegenerative diseases, stresses the importance of an adequate cellular stress defense via functional and efficient protein quality control systems (Dobson, 2003; Morimoto, 2008; Shorter, 2008).

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Abbreviations used: AAC, ADP/ATP-carrier; PMSF, phenylmethylsulfonyl fluoride; ROB, reactive oxygen species; TCA, tricarboxylic acid.

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Recently mitochondrial dysfunction has been recognized as a major factor in the pathogenesis of neurodegenerative diseases (Kwong *et al.*, 2006; Lin and Beal, 2006). Closely related to problems occurring during neurodegeneration, a decline of mitochondrial functions also has been described as an important aspect of aging processes (Navarro and Boveris, 2007). Hence the question arises whether protein aggregation events under stress contribute to functional defects of mitochondria, thereby directly or indirectly favoring pathological processes. In particular, the increased production of ROS, for example, during aging, has been hypothesized to lead to the irreversible damage of mitochondrial proteins (Cadenas and Davies, 2000).

As mitochondria constitute a largely independent intracellular compartment, separated from the cytosol by a double-membrane system, they contain their own set of protein quality control enzymes (Voos, 2009). Due to the endosymbiotic origin of the organelle, these chaperones and proteases closely resemble their bacterial counterparts. A key role is played by mitochondrial Hsp70 (mtHsp70, in the model organism *Saccharomyces cerevisiae* encoded by the gene *SSC1*). mtHsp70 is an essential component of the so-called motor complex that is responsible for the import of mitochondrial preproteins into the matrix compartment (Voos and Röttgers, 2002). Together with its cochaperones Mdj1 and Mge1, mtHsp70 has been shown to mediate the folding of newly imported polypeptides (Horst *et al.*, 1997). Additionally, the mtHsp70 system was shown to bind and stabilize aggregation-prone model polypeptides and to mediate their refolding to the native state, both *in vitro* and after import into isolated organelles (Prip-Buus *et al.*, 1996; Kubo *et al.*, 1999). Furthermore, several mitochondrial proteins have been shown to aggregate if the mitochondrial chaperonin Hsp60 was inactivated (Hallberg *et al.*, 1993; Dubaquié *et al.*, 1998), most likely due to inefficient folding of the newly imported polypeptides. In addition, mtHsp70 together with the Hsp100 family chaperone Hsp78 acts in reactivation of aggregated proteins (Krzewska *et al.*, 2001; von Janowsky *et al.*, 2006). Hsp78, although not an essential protein, thus plays an important role in mitochondrial thermotolerance, as many important processes such as mitochondrial translation or DNA replication are sensitive to temperatures higher than 42°C (Schmitt *et al.*, 1996; Germaniuk *et al.*, 2002).

Chaperones have also been shown to closely cooperate with ATP-dependent proteases in the recognition and removal of damaged proteins. In mitochondria, proteases similar to the bacterial Lon and ClpP proteins have been identified, although the latter is absent in the yeast *S. cerevisiae*. The Lon protease homologue, called Pim1 in yeast, has been shown to degrade misfolded or unfolded reporter proteins (Wagner *et al.*, 1994; von Janowsky *et al.*, 2005). A quantitative analysis of the mitochondrial proteome has demonstrated that proteins with a labile tertiary structure were primary targets of Pim1-mediated degradation (Major *et al.*, 2006), indicating that Pim1 acts as the main quality control protease in the mitochondrial matrix. Moreover, during oxidative stress conditions, the mitochondrial enzymes dihydroxyacid dehydratase (Ilv3) and aconitase (Aco1) were strongly susceptible to degradation by Pim1, presumably because they become unstable after covalent oxidative modification of their Fe/S cluster cofactor (Bota and Davies, 2002; Bender *et al.*, 2010). Surprisingly, Pim1 not only cooperates with mtHsp70 in degradation, but also with Hsp78, which seems to be a unique feature of the mitochondrial compartment (Bateman *et al.*, 2002; Röttgers *et al.*, 2002).

Whereas the components of the mitochondrial protein quality control system and the basic mechanisms by which they operate have been identified, much less is known about the behavior of

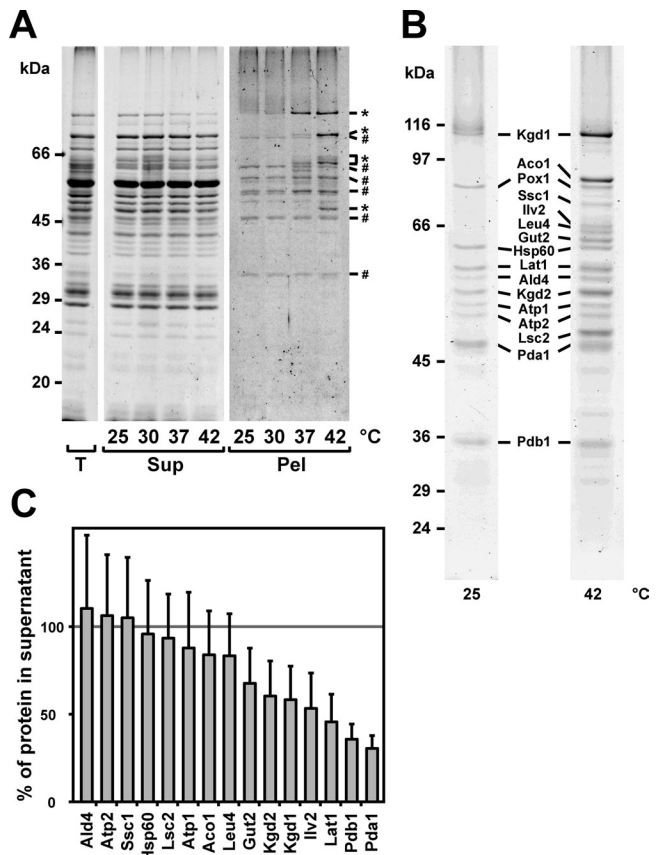
individual endogenous proteins during stress conditions. Most previous experiments were performed using specific artificial reporter proteins, so the results are difficult to generalize due to the inherent differences in the conformational stabilities of individual proteins. In fact, despite the recent identification of several polypeptides that are proteolytically degraded under stress conditions (Bayot *et al.*, 2010; Bender *et al.*, 2010), information about the aggregation behavior of mitochondrial proteins is largely lacking to date. Thus to determine the effects of protein aggregation on mitochondrial function and its potential implication in mitochondrial pathologies, it is of great importance to identify the aggregation-prone protein species and to characterize the conditions that lead to their aggregation. In addition, it is of great interest to define the protective mechanisms that help to keep mitochondrial proteins in a functional state.

In the present work, we used different stress conditions to study protein aggregation in isolated but intact mitochondria from *S. cerevisiae*, an experimental setup closely resembling the *in vivo* situation. Furthermore, by identifying the content of aggregate pellets, we set out to identify specific endogenous proteins that are particularly vulnerable to aggregation. The aggregation behavior of aggregation-prone proteins was characterized in detail. Finally, we investigated the protective effects of mitochondrial protein quality control components, thus establishing a comprehensive picture of deleterious aggregate formation in mitochondria during stress conditions and effective cellular countermeasures.

## RESULTS

### Identification of aggregation-prone mitochondrial proteins

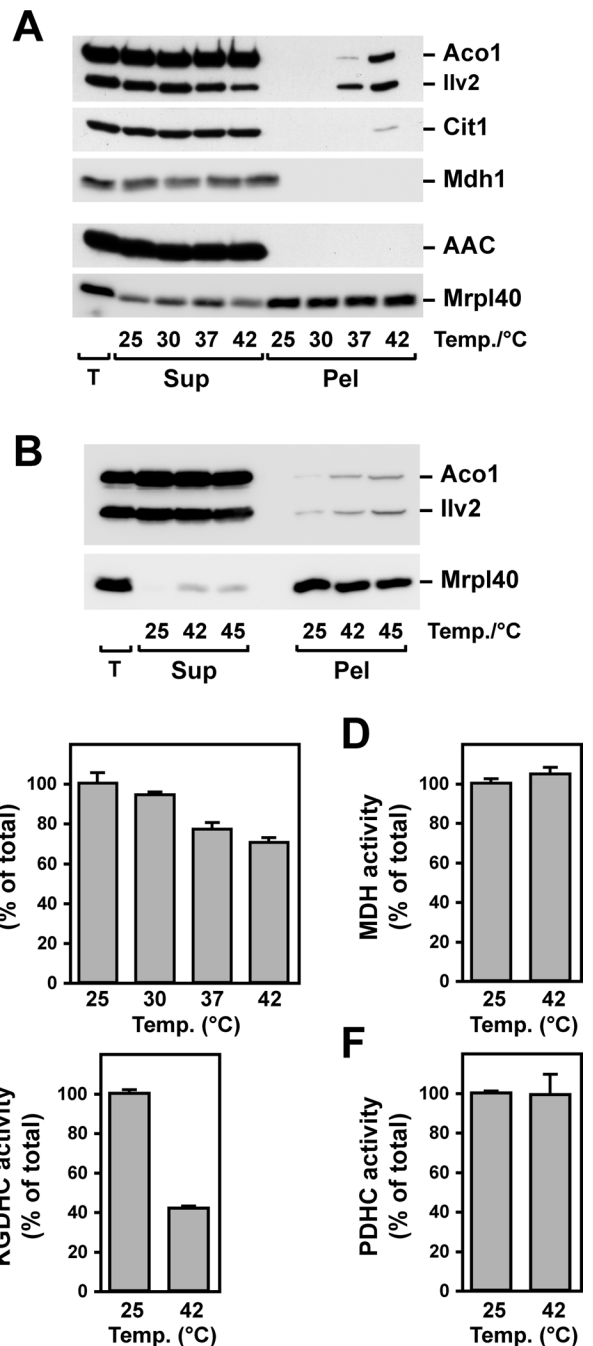
To determine the extent of protein aggregation in an *in vivo*-like situation, we subjected isolated mitochondria from *S. cerevisiae* to a 20-min heat stress treatment. Although a temperature of 30°C can be considered physiological, an increase to 37°C or 42°C represents a mild or strong heat shock, respectively. During the stress treatment, mitochondria were supplied with ATP and NADH to keep them fully energized. Separation of aggregated proteins by a high-velocity centrifugation after *in vivo* heat stress and subsequent detergent lysis is a well-established method that has been used before to characterize protein aggregation in bacteria (Mogk *et al.*, 1999). We used 0.5% Triton X-100 for lysis, which is a mild nonionic detergent that solubilizes lipid membranes but does not denature proteins. Lysates of the treated mitochondria were subjected to centrifugation at 125,000 × *g* in order to separate soluble proteins, which remain in the supernatant, from aggregated polypeptides that sediment to the pellet fraction. After analysis of the pellet fraction by SDS-PAGE, we observed a temperature-dependent increase in the protein amount for at least six protein bands (Figure 1A, marked with \*). These bands presumably represent heat-labile proteins that were present in the soluble fraction at physiological temperatures (25°C or 30°C) but denatured during the stress treatment and formed insoluble aggregates. Most aggregating mitochondrial proteins were in the high-molecular weight range above 50 kDa. On the other hand, a certain set of proteins was found in the pellet fraction independently of the temperature at which the mitochondria were treated (Figure 1A, marked with #). We considered the possibility that some integral membrane proteins might sediment in our aggregation assay due to insufficient solubilization of membranes by detergent lysis. However, immunodecorations with a specific antiserum revealed that the abundant integral membrane protein ADP/ATP-carrier (AAC) is not present in the pellet under all temperature conditions tested (see Figure 2A), so a contamination with membrane proteins can be ruled out. We therefore reasoned that



**FIGURE 1:** Identification of aggregated mitochondrial proteins. (A) Coomassie stain of an SDS gel with lysates of isolated mitochondria from *S. cerevisiae* after treatment at the indicated temperatures and sedimentation of aggregates by ultracentrifugation at  $125,000 \times g$ . Protein bands whose intensity increased with rising incubation temperature are marked with an asterisk (\*); those whose intensity did not change with temperature are marked with #. T, total; Sup, supernatant; Pel, pellet. (B) Aggregates after treatments at 25°C or 42°C were spun down at  $125,000 \times g$ , and the indicated bands identified by mass spectrometry. (C) Quantitative analysis of mitochondrial protein aggregation on 2D-PAGE. Residual spot volume intensities were determined in the soluble fraction after heat treatment and a high-velocity spin and compared with intensities in total mitochondrial lysates. The relative difference of spot intensities in supernatant vs. total (set to 100%) for individual protein species are shown as means of three independent experiments.

extremely large soluble protein complexes, for example, ribosomes, might also be sedimented under the centrifugation conditions applied. Due to their size and density, they should be found in the pellet independently of the temperature conditions. This could be confirmed by Western blotting and immunodecoration with antiserum directed against the mitochondrial ribosomal protein Mrp140 that was present in the pellet fraction in similar amounts at all temperatures (see Figure 2A).

To identify individual heat-labile aggregation-prone mitochondrial proteins, protein bands from the pellet fractions at both 25°C and 42°C were excised from the SDS-PAGE gel and investigated by mass spectrometry analysis. The results are listed in Table 1 (see also Supplemental Materials). Due to the relatively limited number of bands, one-dimensional SDS-PAGE on an 8% acrylamide gel was sufficient for candidate proteins to be resolved (Figure 1B). All examined bands could be identified as mitochondrial proteins, with the exception of peroxisomal fatty-acyl CoA oxidase (Pox1), which



**FIGURE 2:** Aggregation of model proteins during heat stress. (A) Isolated mitochondria were treated at the indicated temperatures, and aggregates were separated by ultracentrifugation at  $125,000 \times g$ . Total lysates (T), supernatants (Sup), and pellets (Pel) were analyzed by SDS-PAGE, Western blotting, and immunodecoration with the indicated specific antisera against mitochondrial proteins. (B) Spheroblasts were created from whole yeast cells to study aggregation in vivo. Cells were then stressed for 30 min at indicated temperatures, and mitochondria were isolated and analyzed as described above. (C-F) Enzyme activity assays of TCA cycle enzymes. Activity of aconitase (C), malate dehydrogenase (D), and alpha-ketoglutarate (E) and pyruvate (F) dehydrogenase complexes were measured after treatment of isolated mitochondria at temperatures ranging from 25°C to 42°C.

most likely reflects a contamination of isolated mitochondria with peroxisomes. As major targets of aggregation, we found eight mitochondrial proteins in the pellet after heat treatment at 42°C that

No.	Protein	Description	UniProt entry	Size* (kDa)	Rel. amount in supernatant (%)
1	Aco1	Aconitate hydratase	P21147	85	84 ± 25
2	Ald4	Aldehyde dehydrogenase	P46367	57	110 ± 42
3	Atp1	F <sub>1</sub> F <sub>0</sub> ATPase complex, subunit $\alpha$	P07251	59	88 ± 32
4	Atp2	F <sub>1</sub> F <sub>0</sub> ATPase complex, subunit $\beta$	P00830	55	106 ± 35
5	Gut2	Glycerol-3-phosphate dehydrogenase	P32191	72	68 ± 20
6	Hsp60	Heat shock protein 60	P19882	61	96 ± 31
7	Ilv2	Acetolactate synthase	P07342	75	53 ± 20
8	Kgd1	$\alpha$ -Ketoglutarate dehydrogenase	P20967	114	58 ± 19
9	Kgd2	Dihydrolipoyl transsuccinylase	P19262	50	60 ± 20
10	Lat1	Dihydrolipoylamide acetyltransferase, subunit E2	P12695	52	46 ± 16
11	Leu4	$\alpha$ -Isopropylmalate synthase	P06208	68	83 ± 24
12	Lsc2	Succinyl-CoA ligase, subunit $\beta$	P53312	47	93 ± 25
13	Pda1	Pyruvate dehydrogenase, subunit $\alpha$	P16387	46	30 ± 7
14	Pdb1	Pyruvate dehydrogenase, subunit $\beta$	P32473	40	36 ± 9
15	Pox1	Acyl-CoA oxidase	P13711	84	n.d.
16	Ssc1	Heat shock protein 70	P12398	71	105 ± 35

\* Molecular weight according to database (<http://www.yeastgenome.org/>). Actual size may vary due to cleavage of mitochondrial targeting signal after import.

**TABLE 1:** Proteins identified in the aggregate pellet after 42°C heat treatment.

could not be detected at all after treatment at 25°C: Aco1, mtHsp70 (Ssc1), acetolactate synthase (Ilv2), alpha-isopropylmalate synthase (Leu4), glycerol-3-phosphate dehydrogenase (Gut2), and the beta subunit of succinyl-CoA ligase (Lsc2) (Figure 1B). In addition, two components of the alpha-ketoglutarate dehydrogenase complex (KGDHC; Kgd1 and Kgd2) were found in the pellet after heat treatment. Although they could also be detected in the pellet fraction at physiological temperatures, their amount was strongly increased at 42°C compared with 25°C (Figure 1B). Apart from the subunits of the KGDHC, we also found subunits of the pyruvate dehydrogenase complex (PDHC; Lat1, Pda1, Pdb1) as prominent proteins in the pellet at 25°C, supporting the notion that large and stable protein complexes sediment during our assay conditions without being in an aggregated state. Other bands in the pellet at 25°C were identified as Hsp60, and F<sub>0</sub>F<sub>1</sub>-ATPase subunits alpha and beta (Atp1, Atp2). Again, these proteins are members of large oligomeric complexes residing in the matrix compartment. In addition, we found the enzyme aldehyde dehydrogenase (Ald4) in the pellet fraction, probably due to its extremely high overall abundance in the mitochondrial matrix. None of these proteins showed an increased amount in the pellet in a temperature-dependent manner.

Considering the absolute protein amount, Aco1 and Kgd1 were the most prominent bands identified in the pellet fraction at 42°C (Figure 1B). However, because both are per se highly abundant proteins, a large protein amount in the pellet might only represent a minor fraction of the total protein present in intact mitochondria. We thus performed a quantitative analysis of the protein amounts in the supernatant before and after heat treatment to determine the specific thermolability of the identified candidate proteins. Although the pellet fraction containing aggregates can be resolved in a one-dimensional SDS gel, quantification of protein band intensities in total mitochondrial lysates was not possible due to limited resolution. We therefore used two-dimensional gel electrophoresis (2D-PAGE), a technique that has been used extensively to quantitatively

analyze endogenous mitochondrial proteins under normal and stress conditions (Major *et al.*, 2006; Bender *et al.*, 2010). Like in the aggregation assay described above, we subjected isolated mitochondria to a heat shock treatment for 20 min at 42°C. The mixture was then depleted from large protein aggregates by a high-velocity spin. The supernatant, containing the soluble fraction, as well as a corresponding sample of total mitochondrial lysate before ultracentrifugation were then subjected to 2D-PAGE to resolve the proteins of interest. The difference between the intensity of an individual protein spot in the supernatant sample and the intensity in the total lysate sample thus reflected the amount of aggregated protein. All spots corresponding to the identified mitochondrial proteins described above (Figure 1B) could be identified by visual comparison with master gels from previous publications from our group (Major *et al.*, 2006; Bender *et al.*, 2010). The relative reduction of protein amounts in the supernatant was calculated from three independent gels as spot volume ratios of supernatant to total sample. The lowest ratios, indicating the highest relative loss from the soluble fraction, were obtained for components of the PDHC and KGDHC (Pda1, Pdb1, Lat1, Kgd1, Kgd2; Figure 1C). The strongest decrease was measured for Pda1, a ratio of 30% indicating that ~70% was found in the pellet. However, because those enzyme complexes sedimented also when incubated at physiological temperatures, the strong decrease in the supernatant can only partially be attributed to aggregation. A substantial temperature-dependent aggregation could be confirmed for the proteins Ilv2 (ratio of 54%) and Gut2 (67%). Intermediate change values were determined for Leu4, Aco1, and Atp1, whereas Lsc2, Hsp60, and Ssc1 remain virtually unchanged (Figure 1C). Although Ssc1, Atp2, and Ald4 were found in the pellet after heat shock, due to their high abundance, the respective ratios were around 100%, indicating that overall activity of these proteins was not impaired even if a minor fraction was aggregating. The majority of mitochondrial proteins thus remain soluble during heat stress, although there were prominent exceptions. Taken



together, both the determination of relative aggregate fractions by comparing total versus soluble protein (Figure 1C) and the specific sedimentation behavior in response to heat treatment (Figure 1B) reveal some primary targets of protein aggregation in mitochondria: *Ilv2*, *Gut2*, *Aco1*, and *Leu4*. All of these proteins are metabolic enzymes, indicating that the respective pathways might be affected by massive protein aggregation.

### Key metabolic enzymes are specifically inactivated by stress

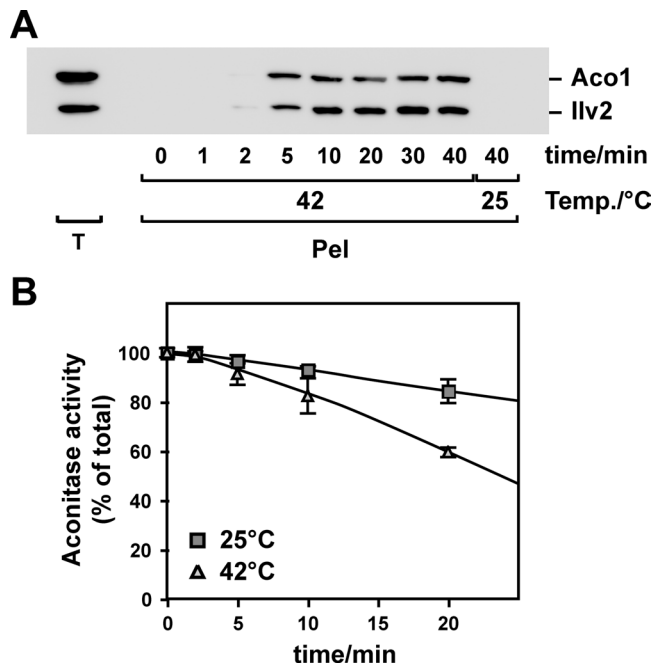
Having determined the content of mitochondrial aggregate pellets after heat shock, we then chose two specific heat-labile proteins as reporter molecules to further investigate the conditions that govern protein aggregation processes in mitochondria. *Aco1* is a major enzyme of the tricarboxylic acid (TCA) cycle, and is one of the most abundant soluble proteins in the mitochondrial matrix. *Ilv2* is also a metabolic enzyme, involved in the synthesis of branched-chain amino acids.

To determine the specific vulnerability of *Aco1* and *Ilv2* to stress treatment, we performed the aggregation assay after treatment at different temperatures ranging from 25°C to 42°C and analyzed total, supernatant, and pellet fractions by Western blotting and detection with specific antisera. Both *Aco1* and *Ilv2* were detected in the pellet only when the sample was heated to 37°C or 42°C (Figure 2A). Thus both candidates indeed aggregated in a temperature-dependent manner. Whereas ~30% of total *Aco1* in the aggregate pellet was only a minor fraction, this protein still represented one of the main aggregating protein species due to its high abundance. In contrast, up to 90% of total *Ilv2* was found in the pellet fraction at 42°C (Figure 2A). Thus *Ilv2* seems to be an extremely heat-labile protein, which became destabilized already at mild heat shock conditions (35% aggregation at 37°C). *Aco1*, on the other hand, remained mostly soluble at 37°C; only ~5% were found in the pellet. These results are in good agreement with the quantitative 2D-PAGE studies described above. For control purposes, we also probed the Western blot membrane with antisera against two other matrix enzymes. Whereas only negligible amounts of citrate synthase (*Cit1*) are found in the pellet, malate dehydrogenase (*Mdh1*) remains completely soluble at all conditions tested (Figure 2A). As a further control, the integral inner membrane protein AAC was never found in the pellet, indicating that pellets were devoid of insufficiently lysed mitochondrial membrane fractions. *Mrpl40* was used as a positive control for the aggregation assay since we observed that ribosomes sedimented irrespectively of the temperature at which mitochondria were treated (Figure 2A). These controls thus verified that the amounts of *Aco1* and *Ilv2* found in the pellet after high-speed centrifugation represented true protein aggregates. Although the purified mitochondria used in these assay represent a functionally intact organelle with a closed membrane, we also tested whether the two reporter proteins *Aco1* and *Ilv2* aggregated in intact cells, representing the true *in vivo* environment. We therefore performed the aggregation assay using yeast spheroblasts, created by treatment of whole yeast cells with zymolyase. This enzyme removes the yeast cell wall, leaving the plasma membrane intact. This procedure was chosen to minimize the time required to isolate the mitochondrial samples after the induction of the aggregation reaction. If spheroblasts were subjected to heat treatment for 30 min at 42°C or 45°C, we observed significant aggregation of both reporter proteins (Figure 2B). Although aggregation rates were considerably lower than during the *in organello* assay, the temperature dependence indicates that aggregation of mitochondrial proteins also occurs in intact cells.

Most of the mitochondrial proteins found to aggregate were enzymatic components of major metabolic pathways like TCA cycle or amino acid synthesis. Presumably, aggregation of metabolic enzymes should result in an overall loss of enzymatic activity, possibly leading to a fatal failure of cellular metabolism. Therefore, we were interested to examine whether the aggregation correlated with a loss of enzymatic activity, and we determined the enzymatic activities of selected aggregation targets in mitochondrial lysates after different heat treatments. Although being a major target of aggregation regarding absolute protein amount, around 70% of *Aco1* remains in the soluble fraction after a 20-min treatment at 42°C (Figure 2A). Indeed, *Aco1* activity under the same conditions was decreased to ~70% compared with a control sample treated at 25°C (Figure 2C), so the loss of enzymatic activity can be attributed to aggregation. Interestingly, *Aco1* activity decreased gradually as the temperature was increased, although massive aggregation could only be observed at 42°C. At 30°C, activity was reduced to 94%, at 37°C, only 77% of *Aco1* was still active. This suggests that *Aco1* aggregation is preceded by loss of enzymatic activity, presumably due to thermal unfolding and inactivation of the Fe/S cluster in the active center. As a control, we also determined the activity of an enzyme that did not aggregate. Indeed, *Mdh1* was still completely active after 20 min at 42°C (Figure 2D).

Some of the most prominent mitochondrial proteins in the pellet after heat shock were components of the PDHC and KGDHC. However, control experiments without heat treatment suggest that both complexes remained assembled during detergent lysis and sedimented at least in part without having aggregated. It thus remained unclear whether these complexes become functionally compromised during heat stress. To clarify this matter, we also investigated the enzymatic activities of both protein complexes. Components of KGDHC, *Kgd1* and *Kgd2*, despite sedimentation already after treatment at 25°C, appeared to form aggregates because the fraction of protein in the pellet still increased if the sample had been heated to 42°C (see Figure 1B). Indeed, when we measured the activity of the complex under the same conditions, the heat-treated sample displayed only 41% of the activity compared with treatment at 25°C (Figure 2E), indicating a certain thermolability of the complex components. In contrast, PDHC remained fully active even after heat treatment at 42°C (Figure 2F). This was also in good agreement with the aggregation assay, where complex components *Pda1*, *Pdb1*, and *Lat1* did not sediment in greater amounts after heat treatment, compared with treatment at physiological temperatures (see Figure 1B). Measuring their enzymatic activities thus confirms the temperature-dependent inactivation of aconitase and KGDHC due to aggregation.

The kinetics of protein aggregation were also studied. Aliquots of isolated mitochondria were incubated at 42°C for several time points ranging from 0 to 40 min. To confirm the heat specificity of aggregation, another sample was incubated for 40 min at 25°C, showing again no aggregation for either *Aco1* or *Ilv2* (Figure 3A). If incubated at 42°C, both proteins aggregate with similar kinetics. There was a short lag period of ~2–4 min, as no protein was found in the pellet at early time points. *Aco1* and *Ilv2* were first detected in the pellet after 5 min of heat treatment. After 10 min, the amount of aggregated protein did not increase substantially any more, showing a critical time frame for aggregation between 5 and 10 min of heat shock (Figure 3A). Furthermore, *Aco1* activity decreased if mitochondria were treated at 42°C after a short lag period (Figure 3B). The comparison of *Aco1* solubility and enzymatic activity again suggests a correlation of thermal inactivation and aggregation of the protein.



**FIGURE 3:** Kinetics of aggregation and decline of enzymatic activities. (A) Aggregation time course. Isolated mitochondria were incubated at 42°C for the indicated time spans, and aggregates were separated by ultracentrifugation. T, total mitochondrial lysate; Sup, supernatant; Pel, aggregate-containing pellet. (B) Decline of aconitase activity was measured in mitochondrial lysates after treatment at 25°C or 42°C for the indicated time spans. Values shown are means  $\pm$  SEM of three independent experiments.

### Oxygen radicals as cause of protein aggregation

Stress through ROS has been described as another major threat to protein integrity aside from heat stress (Cabisco et al., 2000). In fact, it should be noted that *in vivo* heat treatment at 42°C might also cause an elevated level of ROS due to increased radical production within the respiratory chain during those conditions. Because mitochondria are the site of oxidative metabolism, it has been discussed that mitochondrial proteins especially are at risk of being damaged by ROS. Consequently, we examined protein aggregation in mitochondria also under oxidative stress conditions. To induce oxidative stress, isolated mitochondria were treated with either hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or menadione, the latter being a vitamin K<sub>3</sub> metabolite leading to the formation of superoxide radicals (Chaput et al., 1983). One of the model proteins examined in the context of heat stress, Aco1, contains an oxidant-sensitive Fe/S cluster as a prosthetic group, making it also an interesting candidate to investigate its aggregation after ROS treatments. However, both Aco1 and Ilv2 remained completely soluble in wild-type mitochondria at concentrations of up to 20 mM H<sub>2</sub>O<sub>2</sub> or 2 mM menadione at 25°C, with the exception of a marginal amount of Ilv2 in the pellet with menadione treatment (Figure 4, A and B; WT).

Enzymatic mechanisms exist in mitochondria to inactivate toxic oxygen radicals. Mn-superoxide dismutase (Sod2) is the major ROS scavenger of the mitochondrial matrix (van Loon et al., 1986), whereas Cu/Zn-superoxide dismutase (Sod1) is found in the intermembrane space (Sturtz et al., 2001). We thus used mitochondria isolated from yeast strains where the genes for Sod1 or Sod2 had been deleted to increase the toxic effect of oxidative treatments. Under the same stress conditions as before, considerably higher

amounts of both Aco1 and Ilv2 could be found in the pellet using *sod2Δ* mitochondria (Figure 4, A and B; *sod2Δ*). Interestingly, Aco1 was more vulnerable to peroxide stress, whereas Ilv2 aggregation was stronger with superoxide stress. We therefore conclude that mitochondrial ROS levels are physiologically kept in check by scavenging enzymes, reducing the accumulation of protein damage and therefore large-scale aggregation. Deletion of *SOD1*, on the other hand, had no effect on aggregation of either Aco1 or Ilv2 under oxidative stress (Figure 4, A and B; *sod1Δ*), which could be expected due to its location in the intermembrane space.

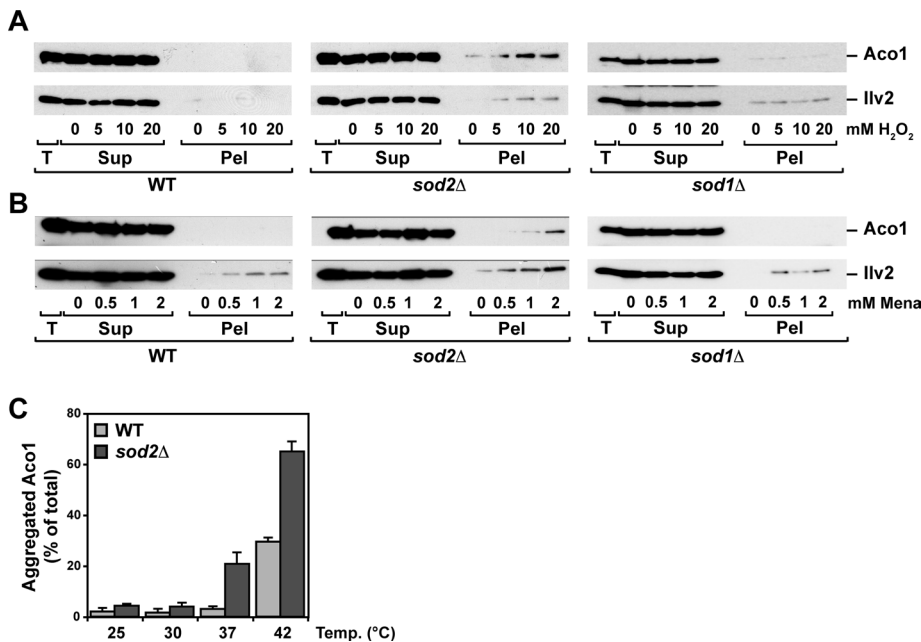
The deletion of *SOD2* alone causes an increased concentration of ROS, even without externally added stressors, because the mitochondrial respiratory chain constantly releases oxygen radicals as a side reaction (Balaban et al., 2005). We therefore used this system to test a combination of oxidative and heat stress with regard to Aco1 aggregation. Isolated mitochondria of wild-type or *sod2Δ* yeast were subjected to temperatures ranging from 25°C to 42°C. While at physiological temperatures, aggregation of Aco1 was only slightly increased in *sod2Δ*; the protein was far more vulnerable in *sod2Δ* mitochondria at 37°C (3% vs. 21% aggregated) or 42°C (29% vs. 65% aggregated). This confirms that a failure to detoxify ROS in the *sod2Δ* background leads to a more harmful effect of heat stress on protein conformation and solubility, significantly enhancing the danger of protein aggregation.

### Protection from aggregation by chaperones and proteases

Mitochondria contain an elaborate network of protein quality control, consisting of chaperones and proteases. Protein quality control is an energy-consuming process, as the relevant chaperones and proteases all have intrinsic ATPase activities. To assess the impact of the protein quality control system on the aggregation of mitochondrial proteins, we therefore first tested the dependence of aggregation on mitochondrial ATP levels. We compared protein aggregation levels in yeast mitochondrial lysates supplied with ATP with corresponding samples where endogenous ATP was depleted by treatment with apyrase. Aggregation of both Ilv2 and Aco1 was indeed increased in ATP-depleted samples at 42°C (Figure 5A), indicating that ATP-dependent processes are needed to secure mitochondrial protein homeostasis. Additionally, without ATP, significantly stronger aggregation levels of Aco1 could be observed already at 37°C, although the majority of Aco1 still remained soluble at this temperature.

As chaperones are supposed to prevent protein aggregation by stabilizing aggregation-prone polypeptides, we also determined the sedimentation behavior of several mitochondrial chaperone proteins. The chaperone protein Ssc1 (the mitochondrial Hsp70) along with its cochaperone Mdj1 could be found in the pellet if mitochondria were treated at 37°C or 42°C, however, only in very small amounts compared with the total sample (Figure 5B). This observation was also in good agreement with the quantitative analysis described before, where no significant reduction of Ssc1 levels due to aggregation could be observed at the same conditions (see Figure 1C). So, the mitochondrial Hsp70 system seems to remain largely functional during heat stress. Additionally, both the disaggregating chaperone Hsp78 and the ROS-scavenger Sod2 remain completely soluble (Figure 5B).

To directly demonstrate the protective effect of protein quality control components, aggregation of the model proteins was then examined in isolated mitochondria from mutant strains in the presence of ATP. Because the major chaperone of the mitochondrial matrix, Ssc1, is an essential protein, we used the temperature-sensitive mutant *ssc1-3*. Before mitochondria were subjected to the



**FIGURE 4:** Aggregation of model proteins during oxidative stress. (A, B) Isolated mitochondria from WT, *sod1Δ* and *sod2Δ* strains were treated at 25°C with the indicated amounts of oxidative stressor ranging from 0 to 20 mM H<sub>2</sub>O<sub>2</sub> (A) or 0 to 2 mM menadione (B), and aggregates were separated by ultracentrifugation at 125,000 × g. Total lysates (T), supernatants (Sup), and pellets (Pel) were analyzed by SDS-PAGE, Western blotting, and immunodecoration with the indicated specific antisera against mitochondrial proteins. (C) Mitochondria from wild-type (WT) or *sod2Δ* yeast strains were treated at the indicated temperatures, and aggregates separated by ultracentrifugation. Pellets were analyzed by SDS-PAGE and Western blotting. Values shown are means ± SEM of the ratio of protein amount in the pellet compared with total mitochondrial lysate.

treatment at different temperatures (20 min at 25–42°C), they were incubated at 37°C for 2 min to inactivate the Ssc1–3 protein. Whereas aggregation of Ilv2 was barely detectable in wild-type mitochondria at 25°C or 30°C, up to 50% of total protein was aggregated in *ssc1–3* mitochondria at the same conditions (Figure 5C). At higher temperatures, the difference was not as pronounced, probably because Ilv2 aggregation levels were high in wild-type mitochondria already. Moreover, although only 1.5% of total Aco1 was aggregated in *ssc1–3* at 30°C, this still represents a significant increase to wild-type, where no aggregated Aco1 could be detected at all (Figure 5C). In addition, when we used mitochondria where the Hsp70 cochaperone Mdj1 had been deleted (*mdj1Δ*), an approximately twofold increase in aggregated Ilv2 compared with wild-type mitochondria could be detected (20% vs. 39% at 25°C and 21% vs. 39% at 30°C; Figure 4D). The surprisingly strong basal aggregation levels in the control mitochondria (WT) in these experiments were probably due to the use of a respiratory-deficient (*rho*<sup>-</sup>) control strain lacking mitochondrial DNA. This was necessary because the *mdj1Δ* strain is also *rho*<sup>-</sup>. On the other hand, no significant difference between control and *mdj1Δ* mitochondria could be detected for Aco1. However, in case of Aco1, the individual experiments using the mutant mitochondria showed a high degree of variation, most probably due to a strongly reduced expression level in *mdj1Δ* mitochondria (data not shown). Taken together, the above experiments show only a moderate protective role of the mtHsp70 system against protein aggregation at higher temperatures, whereas the protective effect was most pronounced at physiological conditions.

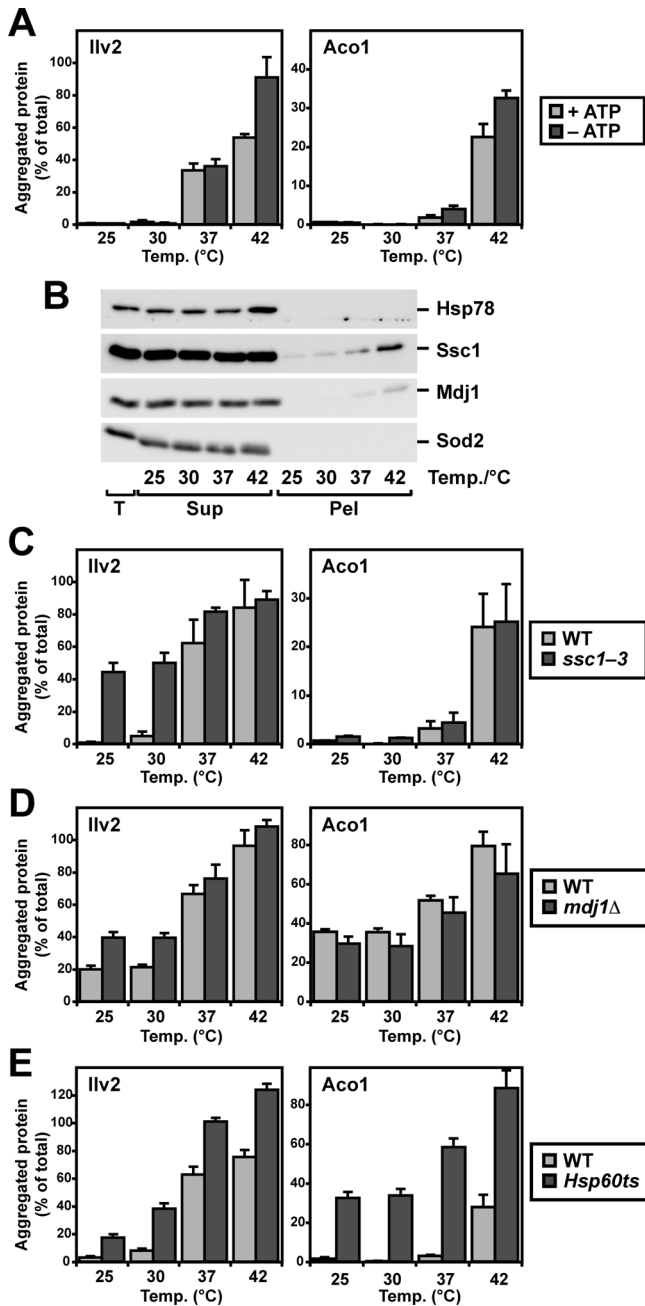
Another chaperone component implicated in de novo folding of mitochondrial proteins is the chaperonin Hsp60. It thus seemed possible that Hsp60 also has a protective effect on aggregation-

prone mitochondrial proteins. Like Ssc1, Hsp60 is an essential protein, so we took advantage of mitochondria from the temperature-sensitive yeast strain *mif4* (Cheng et al., 1989), which carries a mutated Hsp60 allele that can be inactivated by a short heat treatment at 37°C (data not shown). Under these nonpermissive conditions, the amount of aggregated Ilv2 in *mif4* mitochondria was strongly increased compared with wild-type cells at all temperature conditions tested, the protein even aggregating completely if treated at 37°C or 42°C (Figure 5E). Likewise, in the absence of functional Hsp60, ~88% of Aco1 was aggregated at 42°C compared with only 28% in wild-type mitochondria (Figure 5E). Moreover, although virtually no Aco1 protein aggregated at physiological temperatures in the wild-type, around 30% of Aco1 could be found in the pellet at either 25°C or 30°C if Hsp60 was inactivated. It thus seems clear that especially Aco1 but also Ilv2 are protected from aggregation by the Hsp60 chaperonin complex.

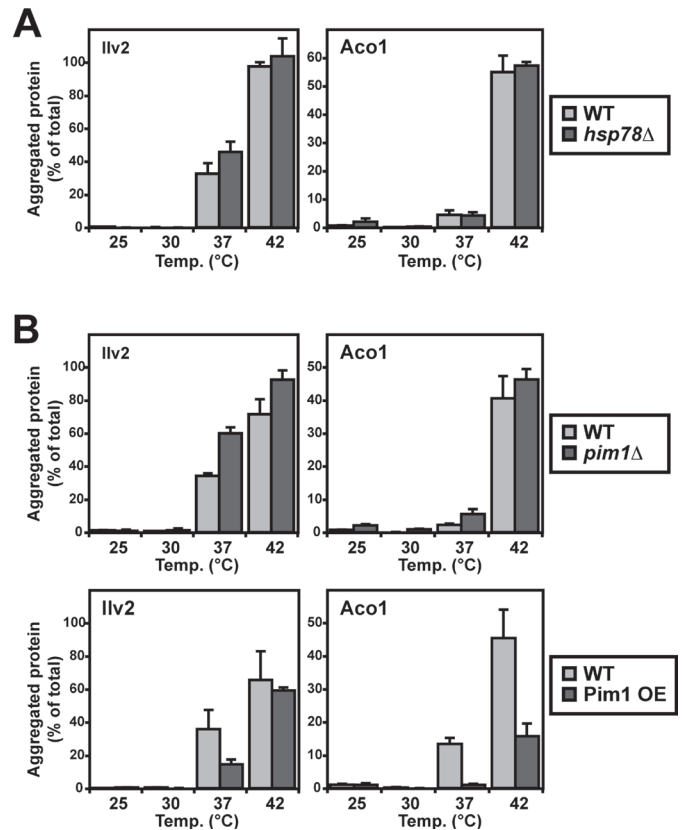
The Hsp100/Clp-like chaperone Hsp78 and its bacterial relative ClpB have been implicated in the recovery of proteins from aggregates (Goloubinoff et al., 1999; Krzewska et al., 2001). We also used mitochondria lacking Hsp78 in our assay to test whether the potential disaggregation activity of

Hsp78 has a general protective effect on our reporter proteins. However, no significant differences in aggregation levels of Aco1 and Ilv2 could be observed between wild-type and *hsp78Δ* mitochondria at all temperatures tested (Figure 6A). We conclude that, under heat stress, the overall benefit of the Hsp78 activity concerning the protection of the model substrates from aggregation was negligible.

Apart from the stabilization of labile protein by chaperones, the removal of terminally damaged polypeptides was suggested to play a role in prevention of aggregation processes. Because Pim1 represents the major ATP-dependent protease of the mitochondrial matrix, we performed the aggregation assay in mitochondria isolated from the deletion strain *pim1Δ*. We observed an approximately twofold increase of aggregation in *pim1Δ* at 37°C compared with wild-type, for both Ilv2 (34% vs. 60%) and Aco1 (2.3% vs. 5.5%). At 42°C, Ilv2 aggregation was also increased from 71% to 92% (Figure 6B, upper panel). It is therefore very likely that proteolytic degradation of destabilized substrates competes with their aggregation, leading to increased aggregation if the protease is deleted. To confirm this hypothesis, we also investigated a yeast strain in which Pim1 was overexpressed from a plasmid-borne copy of the gene. Indeed, aggregation of Aco1 was strongly reduced in this strain, compared with the wild-type (1.5% vs. 14% at 37°C and 21% vs. 60% at 42°C; Figure 6B, lower panel). At 37°C, aggregation of Ilv2 could be reduced from 58% to 20% by overexpressing Pim1, whereas no significant effect on Ilv2 aggregation was observed at 42°C. Degradation of both Ilv2 and Aco1 under stress conditions could also be confirmed by detecting a decrease of soluble protein in the supernatant fractions (data not shown). These experiments corroborate that, under in vivo conditions, the degradation of



**FIGURE 5:** Protection from aggregation by the mitochondrial Hsp70 and Hsp60 chaperone systems. (A) Dependence of aggregation on mitochondrial ATP levels. Isolated mitochondria were lysed and then either supplied with 5 mM ATP (+ATP), or treated with apyrase to deplete endogenous ATP (-ATP). After heat treatment at indicated temperatures, aggregates were spun down by ultracentrifugation, and the relative amount of aggregated protein was determined. (B) Isolated mitochondria were treated at indicated temperatures and then analyzed after ultracentrifugation at 125,000  $\times$  g. Total (T), supernatant (Sup), and pellet (Pel) were subjected to SDS-PAGE, Western blotting, and immunodecoration with specific antisera against the indicated mitochondrial proteins. (C, D) Aggregation in Hsp70 mutants. Isolated mitochondria from wild-type (WT) and either conditional mutant *ssc1-3* (C) or deletion mutant *mdj1* $\Delta$  (D) were analyzed in the aggregation assay. Values shown are means  $\pm$  SEM of the ratio of protein amount in the pellet compared with total mitochondrial lysate. (E) Aggregation in mitochondria from temperature-sensitive strain MIF4 after inactivation of the Hsp60 chaperone.



**FIGURE 6:** Protection from aggregation by other quality control components. (A, B) Isolated mitochondria from wild-type (WT) and deletion mutants *hsp78* $\Delta$  (A) or *pim1* $\Delta$  (B, upper panels) or from yeast overexpressing Pim1 from a plasmid (B, lower panels) were analyzed in the aggregation assay. Values shown are means  $\pm$  SEM of the ratio of protein amount in the pellet compared with total mitochondrial lysate.

destabilized polypeptides plays a major role in prevention of aggregation. In summary, our experiments show that mitochondrial proteins aggregate to a substantial extent if they were challenged by either heat stress or reactive oxygen. As an important aspect of quality control, the proteolytic activity of Pim1 prevents the accumulation of these aggregation-prone polypeptides, resulting in prevention of proteotoxic effects.

The protective effects of both the Hsp70 and Hsp60 chaperone systems on aggregation of mitochondrial proteins raised the question of whether especially newly imported proteins are vulnerable to aggregation, as both chaperone systems are responsible for efficient folding reactions inside the organelle (Cheng *et al.*, 1989; Horst *et al.*, 1997). To differentiate between proteins that were recently imported and proteins that have already adopted their native conformation under steady-state conditions, we synthesized and labeled the aggregation-prone model protein Ilv2 by *in vitro* translation in rabbit reticulocyte lysate in the presence of [<sup>35</sup>S]-methionine. Radioactively labeled Ilv2 can be imported into isolated mitochondria and detected by SDS-PAGE followed by autoradiography. Directly after import, we subjected mitochondria to a 20-min heat treatment at 37°C to induce aggregation, isolated the aggregates by detergent lysis and a high-velocity spin, and finally detected newly imported [<sup>35</sup>S]-Ilv2 by autoradiography and steady-state Ilv2 by immunodecoration with a specific antiserum. Because the amounts of imported labeled preproteins are usually too low to be detected by Western blot, both species of the same protein can be distinguished.



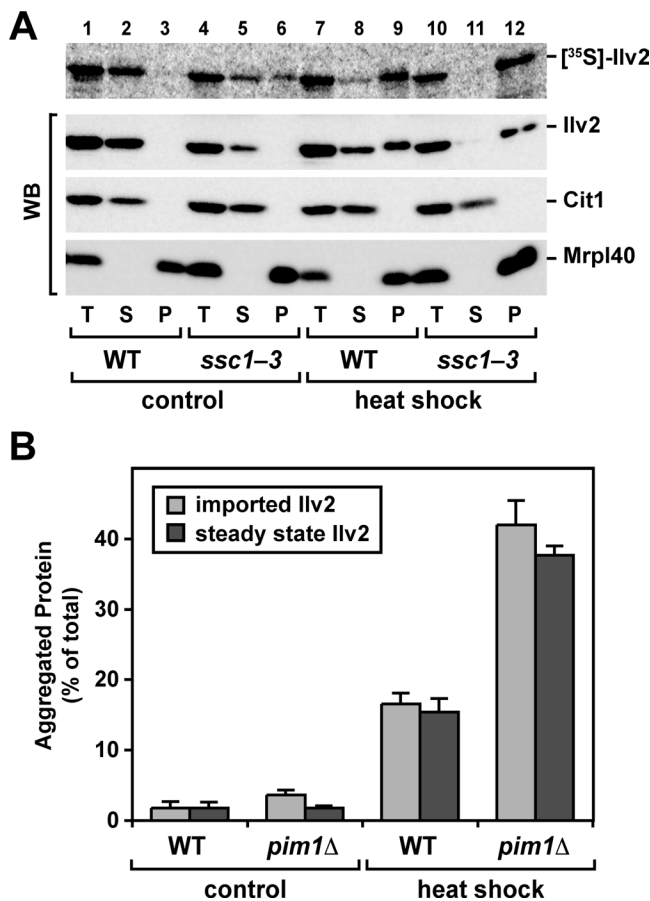
Indeed, whereas a considerable amount of total llv2 remained soluble in wild-type mitochondria following heat treatment, newly imported llv2 was found almost exclusively in the pellet (Figure 7A, lanes 8 and 9). This indicates that newly imported llv2 is more vulnerable to aggregation, possibly because it has not yet folded to its native conformation. Consequently, as unfolded polypeptides should be stabilized by the mtHsp70 system, we also investigated aggregation of newly imported llv2 in *ssc1-3* mitochondria, where the temperature-sensitive mtHsp70 had been inactivated by a short heat treatment. Whereas both newly imported and total llv2 aggregated completely after treatment at 37°C in *ssc1-3* (Figure 7A, lanes 11 and 12), a significant fraction of imported llv2 was found in the pellet even without any heat treatment (Figure 7A, lanes 5 and 6). We thus conclude that Ssc1 not only has a stabilizing effect on fully folded llv2, but is also required to prevent aggregation of still unfolded or incompletely folded protein directly after the import process. In addition, we also investigated aggregation of newly imported llv2 in mitochondria from the *pim1Δ* mutant strain, as also Pim1 was shown to protect steady state llv2 from aggregation (see

Figure 6). However, no significant difference concerning the aggregation behavior between newly imported and steady-state llv2 could be detected in *pim1Δ* (Figure 7B), indicating that the protease is not directly involved in the protection of newly imported proteins against aggregation.

## DISCUSSION

To establish a comprehensive picture of the molecular mechanisms governing protein homeostasis, two different experimental aspects have to be taken into account. First, due to the specific conformational properties of each individual cellular protein, the behavior of endogenous proteins has to be studied in its natural environment. Second, the proteins' dependence on components of cellular protein quality control, in particular under stress conditions, needs to be established. Although the enzymatic components of mitochondrial protein quality control are relatively well-studied (reviewed in Voos, 2009), less evidence about the vulnerability of specific mitochondrial proteins exists, in particular under *in vivo* conditions. In our study, we aimed at a comprehensive characterization of aggregation processes in mitochondria. To maintain a defined environment as close as possible to the natural state, the experiments were performed using intact and energized isolated mitochondria from the model organism *S. cerevisiae*. Performing aggregation assays in organello provides numerous advantages: defined conditions, realistic protein concentrations, and full activity of the protein quality control system. Furthermore, because most mitochondrial proteins are nuclear-encoded and must be imported into the organelle, adaptive mechanisms by changes in cellular protein expression can be ruled out.

Aggregated polypeptides were separated by a high-velocity centrifugation of detergent-lysed mitochondria, a technique that has already been used previously to characterize protein aggregation in bacteria and in yeast mitochondria (Mogk *et al.*, 1999; von Janowsky *et al.*, 2006). Our first goal was to identify mitochondrial proteins that aggregate during various stress conditions. On a general level, our results showed that the majority of mitochondrial proteins remain soluble during heat stress. However, we discovered a set of eight mitochondrial proteins that were abundant in the aggregate pellet as candidates for temperature-induced aggregation. We have found three components of the TCA cycle (Aco1, Kgd1, and Kgd2), two enzymes that are involved in branched-chain amino acid synthesis (llv2 and Leu4), mitochondrial glycerol-3-phosphate dehydrogenase (Gut2), an enzyme of glycerol metabolism, and the major mtHsp70 chaperone (Ssc1). The proteins Aco1 and llv2 also showed a temperature-dependent aggregation behavior in intact cells, essentially ruling out the possibility that the observed aggregation of Aco1 and llv2 is due to the use of isolated mitochondria. The reduced degree of aggregation in intact cells indicates that the overall metabolic state of the cells has at least some influence on the functional activity of the mitochondrial protein quality control system, most probably via the supply of nutrients required for sufficient ATP production. Other mitochondrial proteins that form large enzyme complexes, like pyruvate dehydrogenase, were sedimented under all temperature conditions, indicating that their presence in the pellet is not due to true aggregation. Interestingly, the inactivation of a limited number of mitochondrial proteins while the majority remains unaffected suggests that aggregation is a very specific rather than a random process. Like in a previous study addressing protein aggregation in bacteria (Mogk *et al.*, 1999), we observed an enrichment of high molecular weight proteins (>50 kDa) in the aggregate pellets. It may be speculated that large proteins with higher structural complexity are more vulnerable to partial unfolding that may result in



**FIGURE 7:** Comparison of the aggregation of newly imported and steady-state llv2. (A) Radioactively labeled [<sup>35</sup>S]-llv2 was imported into isolated wild-type (WT) or *ssc1-3* mitochondria, and its aggregation behavior was investigated directly after import control) or following a 20-min heat treatment at 37°C (heat shock). The solubility of endogenous steady-state llv2 was examined by Western blotting and immunodecoration with a specific antiserum (WB). As further controls, the soluble matrix enzyme Cit1 and the ribosomal protein Mrpl40 were also detected by immunodecoration. T, total; S, supernatant; P, pellet. (B) Quantification of the percentage of total newly imported or steady-state llv2 found in the aggregate pellet in wild-type (WT) or *pim1Δ* mitochondria.

aggregation. Interestingly, bacterial homologues of Aco1 and Kgd1 were also shown to aggregate at temperature-stress conditions like incubation at 45°C (Mogk *et al.*, 1999). The bacterial homologue of Ilv2 was not identified as an aggregation-prone protein. However, its specific stability under heat stress was not determined, so a certain sensitivity to aggregation cannot be ruled out. Overall, respective mitochondrial and bacterial proteins appear to behave similarly during heat stress conditions. Taken together, our results indicate that important metabolic pathways in mitochondria can become compromised due to the aggregation of respective key components.

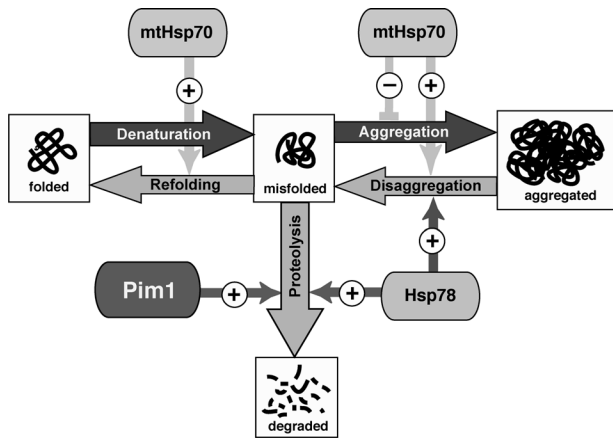
As a first approach, our analysis was aimed at the identification of abundant aggregation candidates. It is expected that the described negative effects on certain metabolic processes represent only the tip of the iceberg because the behavior of most proteins of low abundance, in particular regulative components, could not be characterized so far. However, the decrease of metabolic activity can have a profound impact on the cellular level. It has been described that a decrease of Aco1 activity is a major hallmark of aging in animal mitochondria (Yan *et al.*, 1997; Yarian *et al.*, 2006). Our experiments with yeast mitochondria indicate that aggregation processes contribute to the decline of aconitase function and activity. Similarly, the activity of KGDHC was reduced after heat shock, at least partially, due to protein aggregation. Interestingly, it was reported that KGDHC activity was also reduced in brain mitochondria from patients suffering from Alzheimer's disease (Huang *et al.*, 2003), indicating a possible role of KGDHC components' aggregation in the pathogenesis of this disorder. Taken together, our observations emphasize that aggregation of mitochondrial enzymes plays a role in the development of mitochondrial dysfunction in neurodegenerative diseases and during aging.

As another important aspect of mitochondrial stress, we also examined the effects of ROS because these radicals lead to chemical modifications and inactivation of polypeptides. This is of particular importance because oxygen radicals and mitochondrial dysfunction have been described as a major causative agent in the development of several human diseases as well as aging processes (Lin and Beal, 2006). In general, no significant overall effect of oxidative stress on protein solubility was detected (data not shown). However, we found that both Ilv2 and Aco1 aggregated after treatment with oxidative stressors like menadione or hydrogen peroxide, correlating with previous results that aconitase is readily inactivated by oxygen radicals both in yeast and in mammalian mitochondria (Bota and Davies, 2002; Bender *et al.*, 2010). Because Aco1 contains an oxidant-sensitive Fe/S cluster as a prosthetic group (Nulton-Persson and Szweda, 2001), it is conceivable that its structural integrity is altered or compromised during oxidative stress, resulting in misfolding and aggregation. Interestingly, ROS-induced aggregation of these proteins was strongly increased if Sod2, the major radical scavenger of the mitochondrial matrix, was deleted. Our results correlated well with a study that identified both Aco1 and Ilv2 as mitochondrial proteins that show high levels of carbonylation, a typical marker of oxidative protein modification, if Sod2 is absent (O'Brien *et al.*, 2004). Our observations showed that Sod2 is able to detoxify externally added ROS and therefore protects mitochondrial proteins from resulting destabilization and aggregation. Detoxification of superoxide radicals by Sod2 can therefore be regarded as a major defense mechanism in securing protein homeostasis under oxidative stress conditions.

The fact that during stress conditions important metabolic pathways in mitochondria are affected by aggregation of their enzymatic components raises the question which components contribute to the maintenance of mitochondrial homeostasis and func-

tionality under adverse conditions. We have therefore examined the roles of the major components of the mitochondrial protein quality control system in the matrix compartment. The fact that a depletion of ATP levels increased aggregation suggests a protective effect of the ATP-dependent chaperone and protease components. Hsp70-type chaperones generally exhibit a high affinity to unfolded polypeptide segments exposing hydrophobic amino acid residues, thereby contributing to the stability of damaged substrate proteins (Bukau *et al.*, 2006). Our experiments demonstrated that the soluble matrix chaperone mtHsp70, or Ssc1 in yeast (Voos and Röttgers, 2002), is able to inhibit, at least partially, the aggregation of the reporter substrate Ilv2. This confirms observations where artificial reporter proteins like luciferase were protected by mtHsp70 from denaturation and refolded after heat treatment *in vitro* (Kubo *et al.*, 1999; Liu *et al.*, 2001). Due to the particular mechanism of protein import, mitochondrial proteins enter the matrix in an unfolded conformation (Schwartz *et al.*, 1999). Any delay in the subsequent folding steps would render newly imported preproteins susceptible to denaturation and/or aggregation. Indeed, newly imported Ilv2 polypeptides showed a slightly increased tendency to form aggregates at mild temperature stress, an effect that was significantly enhanced in mtHsp70-defective mutant mitochondria. Because mtHsp70 is the first chaperone a newly imported polypeptide chain encounters (Horst *et al.*, 1997; Strub *et al.*, 2000), a defective chaperone function would directly impede the folding reaction, escalating the problem of aggregation. Also in bacteria, the *Escherichia coli* Hsp70 homologue DnaK is prominently involved in prevention of protein aggregation (Mogk *et al.*, 1999; Tomoyasu *et al.*, 2001). In particular, at 42°C in the absence of DnaK, aggregation rates were significantly increased (Mogk *et al.*, 1999). However, in this study, the "holding" effect of the mitochondrial Hsp70 system was most pronounced at physiological conditions. At higher temperatures, this protective effect was either overcome by the larger amount of aggregated polypeptides or by a certain heat sensitivity of the chaperone itself. The Hsp60 chaperone system has also been shown to be prominently involved in the folding of mitochondrial proteins after import has been completed. Interestingly, Hsp60 seemed to have an even higher influence on the aggregation propensity of mitochondrial proteins at all temperatures as the mtHsp70 system, indicating that Hsp60 is a prominent component of the mitochondrial protein quality control system. As was already shown in screening experiments for mitochondrial substrates (Dubaquíe *et al.*, 1998), the mitochondrial enzyme Aco1 was strongly dependent on the activity of Hsp60. Interestingly, due to the large size of Aco1, with a molecular mass of ~82 kDa, a nonconventional mechanism of the Hsp60 complex is needed for the assistance of Aco1 folding (Chaudhuri *et al.*, 2001).

Another chaperone component that has been described in mitochondria and has been implicated in aggregation reactions is the AAA+ chaperone Hsp78, a homologue of the bacterial ClpB protein (Krzewska *et al.*, 2001; von Janowsky *et al.*, 2006). Generally, it is believed that ClpB and its homologues restore protein function by assisting the resolubilization and refolding of formerly aggregated polypeptides (Goloubinoff *et al.*, 1999; Krzewska *et al.*, 2001). Based on this hypothesis, no direct effect of Hsp78 on protein aggregation would be expected. Indeed, only a very small difference could be detected regarding the aggregation of Ilv2 in WT and *hsp78Δ* mitochondria. This correlates with other studies in bacteria, where the homologue ClpB also could not prevent aggregation as such but rather executes the resolubilization of aggregates together with the Hsp70 system (Mogk *et al.*, 1999). This protective



**FIGURE 8:** Overview of the protein quality control system of the mitochondrial matrix. During stress conditions, proteins become unfolded and subsequently aggregate because of irregular intermolecular interactions of exposed hydrophobic patches. mtHsp70 promotes refolding of misfolded proteins, prevents their aggregation, and cooperates with the ClpB homologue Hsp78 in disaggregation. The ATP-dependent protease Pim1 degrades misfolded proteins to protect them from aggregation, a reaction, which is assisted also by the action of Hsp78.

effect of the disaggregation activity of Hsp78 on mitochondrial functions in vivo has been well established. Hsp78 confers thermotolerance to mitochondria by restoring heat-sensitive processes like mitochondrial translation or DNA replication (Schmitt *et al.*, 1996; Germaniuk *et al.*, 2002), and it assists the chaperone activity of mtHsp70 (von Janowsky *et al.*, 2006). Our results suggest that also amino acid synthesis and the TCA cycle are at least partially sensitive to heat, which raises the question of whether these pathways might be recovered by Hsp78-dependent disaggregation.

Proteolysis is suggested to be a major process that prevents the accumulation of damaged polypeptides before they can form aggregates (Luo and Le, 2010). In our hands, the absence of the ATP-dependent protease Pim1, localized in the matrix of yeast mitochondria, increased aggregation of both model proteins, whereas overexpression of Pim1 drastically reduced the aggregation of both Ilv2 and Aco1. These observations suggest that proteolysis and ag-

gregation are competing processes. In this model, Pim1 would recognize misfolded proteins that cannot be stabilized by the Hsp70 system anymore, and subsequently degrade them before they can form aggregates. This conclusion is supported by the finding that, in case of Ilv2, despite a general dependence of aggregation on the presence of Pim1, no significant difference in the aggregation rates of newly imported and steady-state polypeptides could be detected. The protective effect of Pim1 is most probably based on its ability to recognize unstructured segments of its substrates (von Janowsky *et al.*, 2005), which is the basis for the initiation of its proteolytic activity on the substrate polypeptide. Moreover, a functional cooperation of Pim1 with Hsp70 has been reported, indicating that binding of unfolded proteins is followed by their degradation if refolding fails (Wagner *et al.*, 1994). In addition, Pim1 has been discussed as the primary weapon against oxidatively modified proteins (Bota *et al.*, 2005; Bayot *et al.*, 2010; Bender *et al.*, 2010). ROS-dependent damages in proteins are mostly covalent modifications like carbonylation, where refolding to the functional state is impossible. The results regarding the effect of Pim1 on aggregation are in good agreement with results from studies aimed at the identification of the targets of proteolytic degradation in mitochondria. Aco1 has formerly been reported to be a target of the Pim1/Lon protease both in yeast and human mitochondria, and also Ilv2 was found to be a substrate (Bota and Davies, 2002; Major *et al.*, 2006).

In summary, we thus propose a specific scenario for the maintenance of mitochondrial protein homeostasis (Figure 8). As a primary event, the direct interaction of damaged proteins with mtHsp70 retains them in the soluble state, whereas Pim1 acts like a quality control valve during stress conditions, degrading proteins that the Hsp70 system is not able to protect from aggregation. Hsp78 then acts as a clean-up chaperone, restoring the function of mitochondrial proteins that have aggregated because they have escaped the efforts of stabilizing chaperones. These components of mitochondrial protein quality control therefore form a cooperative functional network that represents a crucial aspect in securing the functional integrity of the organelle.

## MATERIAL AND METHODS

### Yeast strains, plasmids, and isolation of mitochondria

*S. cerevisiae* strains used are listed in Table 2. Strain KRY07 was constructed by transformation of *KanMX6* kanamycin resistance

Strain	MAT	Genotype	Source
BY4741	a	<i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	EUROSCARF
<i>pim1Δ</i>	a	BY4741 <i>pim1Δ::kanMX6</i>	EUROSCARF
<i>hsp78Δ</i>	a	BY4741 <i>hsp78Δ::kanMX6</i>	EUROSCARF
<i>sod1Δ</i>	a	BY4741 <i>sod1Δ::kanMX6</i>	EUROSCARF
<i>sod2Δ</i>	a	BY4741 <i>sod2Δ::kanMX6</i>	EUROSCARF
PK82	alpha	<i>his4-713 lys2 ura3-52 leu2-3,112 trp1</i>	Voos <i>et al.</i> , 1993
PK83	alpha	<i>ade 2-101 lys2 ura 3-52 leu 2-3,112 trp 1 ssc1-3(LEU2)</i>	Voos <i>et al.</i> , 1993
KRY01	a	<i>ura3-5, lys2-801 ade2-101 trp1-Δ63 his1-Δ200 leu2-Δ1 rho-</i>	Röttgers <i>et al.</i> , 2002
KRY07	a	<i>ura3-52 lys2-801 ade2-101 trp1-Δ63 his1-Δ200 leu2-Δ1 rho-mdj1Δ::kanMX4</i>	This study
JK9-3d	alpha	<i>leu2-3112 ura3-52 rme1 trp1 his4 GAL+ HMLa</i>	Cheng <i>et al.</i> , 1989
MIF4	alpha	<i>leu2-3112 ura3-52 rme1 trp1 his4 GAL+ HMLa hsp60Δ::TRP1 pYcPLac-mif4(LEU2)</i>	Cheng <i>et al.</i> , 1989

**TABLE 2:** Yeast strains used in this study.

cassette with flanking regions of the *MDJ1* open reading frame, generated by PCR from genomic DNA of a *mdj1Δ::kanMX6* strain obtained from EUROSCARF (Frankfurt, Germany), and homologous recombination. Pim1 was overexpressed from plasmid pSRX4a, containing the *PIM1* open reading frame under the control of a Gal promoter (constructed from pYES2.1 vector; Invitrogen, Carlsbad, CA). Mitochondria were isolated by differential centrifugation according to published procedures (Ryan *et al.*, 2001). Radiolabeled [<sup>35</sup>S]-IIV2 was imported as described (Major *et al.*, 2006).

### Aggregation assay

A total of 150 μg isolated mitochondria was resuspended in 600 μl of resuspension buffer (250 mM sucrose; 10 mM MOPS-KOH, pH 7.2; 80 mM KCl; 5 mM MgCl<sub>2</sub>; 3 mM ATP; 4 mM NADH). A 100-μl total sample was taken, and mitochondria were reisolated by centrifugation. From the remaining solution, four 100-μl samples were taken and incubated for 20 min at temperatures indicated or at 25°C in the presence of varying concentrations of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, 0–20 mM) or menadione (0–2 mM). After centrifugation for 10 min at 21,000 × *g* and 4°C, mitochondrial pellets were lysed in 100 μl of lysis buffer A (0.5% Triton X-100; 30 mM Tris, pH 7.4; 200 mM KCl; 5 mM EDTA; 0.5 mM phenylmethylsulfonyl fluoride [PMSF]; protease inhibitors) and then subjected to a high-velocity spin at 125,000 × *g* for 30 min at 4°C. Supernatants were either discarded, or, if indicated, precipitated with TCA, whereas pellets were re-extracted by vigorous shaking with 100 μl lysis buffer A and then centrifuged again at 125,000 × *g* for 30 min at 4°C. Total, supernatant, and pellet samples were then analyzed by SDS-PAGE, and, if indicated, Western blotting and immunodecoration with specific antisera against mitochondrial proteins. Chemiluminescence signals were detected using the LAS-4000 mini camera (Fujifilm, Stamford, CT) and quantified with MultiGauge software (Fujifilm). For statistical analysis, means and standard errors were calculated from three individual experiments.

To test the effect of ATP depletion on aggregation of mitochondrial proteins, 150 μg isolated mitochondria were lysed directly in 300 μl of lysis buffer B (0.5% Triton X-100; 30 mM Tris, pH 7.4; 150 mM KCl; protease inhibitors) supplied with either 5 mM ATP and 5 mM MgCl<sub>2</sub> (+ATP) or 5 mM EDTA and 0.01 U/μl apyrase (–ATP). A 50-μl total sample was precipitated with TCA, while four more 50-μl samples were incubated for 10 min at 25°C for ATP depletion, and then for 20 min at temperatures indicated. Aggregation assay was then finished by ultracentrifugation and SDS-PAGE as described above.

To analyze protein aggregation *in vivo*, yeast cells from mid-log phase were first resuspended in 1 ml of TEB buffer (200 mM Tris, pH 8.0; 20 mM EDTA; 1% [vol/vol] β-mercaptoethanol) and incubated for 10 min at 30°C with shaking, then resuspended in 1 ml of SPM buffer (1.2 M sorbitol; 50 mM KPi, pH 7.3; 1 mM MgCl<sub>2</sub>) containing 0.3 mg/ml Zymolyase and incubated for 60 min to create spheroblasts. Cells were then reisolated and resuspended in SPM buffer, and 100-μl aliquots were treated at indicated temperatures (heat stress). Finally, cells were lysed by repeated pipetting in SET buffer (0.2 M sorbitol; 50 mM Tris, pH 7.5; 1 mM EDTA), and mitochondria were isolated by centrifugation for 10 min at 12,000 × *g* and 2°C. Mitochondria were then lysed, centrifuged, and subjected to SDS-PAGE as described above.

### Mass spectrometry

For identification of aggregated mitochondrial proteins, pellets obtained by the aggregation assay at 25°C or 42°C were subjected to

SDS-PAGE on 8% acrylamide gels. Bands of interest were cut out with a scalpel and then digested with Trypsin Profile IGD kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. For analysis, peptides eluted from acrylamide gels were separated using an Ultimate 3000 LC system (Dionex-LC Packings, Idstein, Germany). Samples were loaded onto a monolithic trapping column (PepSwift, 200 μm × 5 mm) with the loading pump of the system operating at 10 μl/min, and 0.1% heptafluorobutyric acid in water used as mobile phase. After 5 min, valve was switched, and the sample was eluted onto the analytical separation column (PepSwift monolithic capillary column, 200 μm × 50 mm), using a flow rate of 500 nl/min. The mobile phases used were H<sub>2</sub>O/0.1% formic acid (vol/vol) for buffer A and 100% ACN/0.1% Formic acid (vol/vol) for buffer B. Peptides were resolved by gradient elution using a gradient of 5–50% buffer B >20 min, followed by a gradient of 50–90% buffer B >1 min. After 5 min at 90% buffer B, the gradient returned to 5% buffer B preparing for the next run. Column effluent was monitored using a 3 nl UV flow cell (214 nm).

Mass spectrometric analyses were done via online ESI-MS/MS using a HCTUltra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). All measurements were carried out in positive ion mode. MS-spectra were acquired in standard-enhanced mode between 300 and 2000 *m/z* at a rate of 8100 *m/z*/s. Fragmentation of peptides from MS-spectra using CID was done in Auto-MS2 mode, selecting precursor ions according to the following parameters: number of precursor ions = 5, minimal ion intensity = 10000, ion excluded after 2 spectra, exclusion release after 1 min. MS2 data acquisition was done in ultrascan mode with a scan range of 50–3000 *m/z* at a scan speed of 26,000 *m/z*/s.

Raw MS data for each LC run were processed using DataAnalysisTM version 4.0. The spectrum was screened for compounds using the software's AutoMS/MS search feature applying following parameters: intensity threshold = 10,000; max number of compounds = 500; retention time = 0.4. Identified compounds were subsequently deconvoluted and exported for protein database comparison with BioTools version 3.1. In BioTools, the exported compounds were run against an in-house SwissProt v51.6 database using the Mascot 2.2.02 algorithm. The searches were carried out using the following parameters: enzyme = trypsin; missed cleavages = 1; taxonomy = all entries; variable modifications = oxidation (M); peptide tolerance = 300 ppm; MS/MS tolerance = 1.1 Da; significance threshold *p* = 0.01.

### 2D-PAGE

For the total sample, 100 μg isolated mitochondria were lysed in lysis buffer (0.5% Triton X-100; 30 mM Tris, pH 7.4; 200 mM KCl; 5 mM EDTA; 0.5 mM PMSF; protease inhibitors) and then precipitated with TCA. For the supernatant sample, 100 μg isolated mitochondria were first resuspended in resuspension buffer (250 mM sucrose; 10 mM MOPS-KOH, pH 7.2; 80 mM KCl; 5 mM MgCl<sub>2</sub>; 3 mM ATP; 4 mM NADH), then heat treated for 20 min at 42°C, lysed in lysis buffer and subjected to ultracentrifugation at 125,000 × *g* for 30 min. Supernatants were then precipitated like the total sample. Both pellets were analyzed by 2D-PAGE as described (Bender *et al.*, 2010).

### Enzymatic assays

To determine enzymatic activities in mitochondrial lysates, isolated mitochondria were resuspended in resuspension buffer (250 mM sucrose; 10 mM MOPS-KOH, pH 7.2; 80 mM KCl; 5 mM MgCl<sub>2</sub>) in varying amounts (30 μg per reaction for malate dehydrogenase assay, 80 μg for other assays). Suspensions of mitochondria were



then incubated at temperatures indicated for 20 min. After centrifugation for 10 min at 21,000 × g and 4°C, mitochondrial pellets were lysed in homogenization buffer (0.5% Triton X-100, 50 mM Tris, pH 7.4). Mitochondrial lysates were then added to 1.4-ml quartz cuvettes containing 1 ml of reaction mixture followed by thorough mixing of the reaction. Reaction mixtures were 50 mM Tris, pH 7.4; 5 mM sodium citrate; 0.6 mM MnCl<sub>2</sub>; 0.2 mM NADP<sup>+</sup>; 0.1 mg/ml isocitrate dehydrogenase (aconitase activity assay), or 50 mM Tris, pH 7.4; 0.5 mM oxalacetic acid, 0.2 mM NADH (malate dehydrogenase activity assay), or 50 mM KCl, 30 mM Tris, pH 7.4; 0.2 mM MgCl<sub>2</sub>; 5 mM alpha-ketoglutarate; 0.3 mM thiamine pyrophosphate; 0.3 mM coenzyme A; 0.4 mM NAD<sup>+</sup> (KGDHC activity assay), or 50 mM KCl, 30 mM Tris, pH 7.4; 0.2 mM MgCl<sub>2</sub>; 5 mM sodium pyruvate; 0.3 mM thiamine pyrophosphate; 0.3 mM coenzyme A; 0.4 mM NAD<sup>+</sup> (PDHC activity assay). Enzymatic activities were measured in a Beckman DU-640 Spectrophotometer by either following the increase in NADPH (aconitase) or NADH (KGDHC, PDHC) absorption, or the decrease in NADH absorption (MDH) at 340 nm. Means and standard errors of at least three individual experiments were calculated.

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