

Reactivation of protein aggregates by mortalin and Tid1—the human mitochondrial Hsp70 chaperone system

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Abstract The mitochondrial 70-kDa heat shock protein (mtHsp70), also known in humans as mortalin, is a central component of the mitochondrial protein import motor and plays a key role in the folding of matrix-localized mitochondrial proteins. MtHsp70 is assisted by a member of the 40-kDa heat shock protein co-chaperone family named Tid1 and a nucleotide exchange factor. Whereas, yeast mtHsp70 has been extensively studied in the context of protein import in the mitochondria, and the bacterial 70-kDa heat shock protein was recently shown to act as an ATP-fuelled unfolding enzyme capable of detoxifying stably misfolded polypeptides into harmless natively refolded proteins, little is known about the molecular functions of the human mortalin in protein homeostasis. Here, we developed novel and efficient purification protocols for mortalin and the two spliced versions of Tid1, Tid1-S, and Tid1-L and showed that mortalin can mediate the *in vitro* ATP-dependent reactivation of stable-preformed heat-denatured model aggregates, with the assistance of Mge1 and either Tid1-L or Tid1-S co-chaperones or yeast Mdj1. Thus, in addition of being a central component of

the protein import machinery, human mortalin together with Tid1, may serve as a protein disaggregating machine which, for lack of Hsp100/ClpB disaggregating co-chaperones, may carry alone the scavenging of toxic protein aggregates in stressed, diseased, or aging human mitochondria.

Keywords Mitochondrial Hsp70 · Tid1 · Hep1 · Ssc1 · Disaggregation

Introduction

The 70-kDa heat shock proteins (Hsp70s) are ubiquitous molecular chaperones, which are present in all the ATP-containing compartments in the eukaryotic cell, where they perform diverse functions all with a common denominator: they use the energy of ATP hydrolysis to unfold, at least partially, various protein substrates (Goloubinoff and De Los Rios 2007; Sharma et al. 2010). The bacterial chaperone DnaK has long served as a prototype for the study of Hsp70 chaperones and its investigation provided important insights into the structure and function of this large group of proteins (Swain et al. 2007). Three types of chaperone activities have been described for bacterial DnaK: (1) passively preventing the aggregation of artificially unfolded proteins (holding function). (2) ATP-dependent refolding of chemically unfolded proteins to their native forms (Szabo et al. 1994). (3) The ATP-dependent reactivation of stable protein aggregates into native proteins. The last two functions reflect the ability of this chaperone system to act as a polypeptide unfoldase. In this capacity, Hsp70 specifically recognizes misfolded polypeptides, bind them with high affinity, uses the energy of ATP hydrolysis to unfold them, and releases low-affinity

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unfolded products that in turn can spontaneously refold to the native state (Sharma et al. 2010). In vitro, DnaK can perform this function when assisted by its two co-chaperones, a J-domain-containing protein (either DnaJ, CbpA, or DjlA; Genevax et al. 2007) and the nucleotide exchange factor GrpE (Sharma et al. 2009). Specificity of the chaperone system is conferred primarily by the DnaJ co-chaperone, which recognizes and binds to a misfolded polypeptide (Hinault et al. 2010), followed by the binding of the polypeptide to a “low-affinity” DnaK, which becomes entrapped upon hydrolyzing ATP. GrpE, acting as an ADP release factor, accelerates discharge of the unfolded polypeptide and spontaneous refolding to the native state (Sharma et al. 2010).

Yeast mitochondria harbor three Hsp70 homologues named Ssc1, Ssq1, and Ecm10 (for sequence alignments see Supplemental Fig. 1). Ssc1 was shown to be a key player in mediating protein import into the mitochondrial matrix. This particular function of the chaperone occurs through association of the ATP-bound state to the TIM23 import channel, by way of the specific protein anchors, Tim44 and PAM16/18. Dissociation of ADP-bound Ssc1 from the anchors takes place as soon as it has locked onto an incoming polypeptide (De Los Rios et al. 2006; D’Silva et al. 2004; Neupert and Herrmann 2007). Ssc1 plays an additional important role in mediating the refolding of matrix-localized proteins, a function which is carried out with the help of two matrix-localized co-chaperones Mdj1 and Mge1, which are homologous to the *Escherichia coli* DnaJ and GrpE, respectively (Deloche et al. 1997; Horst et al. 1997; Liu et al. 2001; Rowley et al. 1994) (Supplemental Fig. 2). Using purified proteins, it was shown that Ssc1 can mediate ATP-dependent disaggregation and proper refolding of stably misfolded reporter proteins, when supplemented with bacterial DnaJ and GrpE (De Los Rios et al. 2006). Ssq1 is known to play a role in the biogenesis of iron sulfur clusters and the function of Ecm10 is still unclear (Baumann et al. 2000; Dutkiewicz et al. 2003).

The human Hsp70 family consists of eight members that differ in their amino acid sequences, expression levels, and cellular localizations (Daugaard et al. 2007). Six Hsp70 chaperones are located mainly in the cytosol and in the nucleus; one Hsp70 is located in the lumen of the ER and mortalin and is found in the mitochondrial matrix. Thus, in human mitochondria, a single Hsp70, mortalin, must take over all functions that are performed by its three homologues in yeast mitochondria (Bhattacharyya et al. 1995; Daugaard et al. 2007; Mizzen et al. 1989; Wadhwa et al. 1993). Mortalin shares high identity with other members of the Hsp70 family, including *E. coli* DnaK (51%) and *Saccharomyces cerevisiae* Ssc1 (65%) (Supplemental Fig. 1). Interestingly, mortalin was also detected in extra-mitochondrial compartments, which reflects the ability of

this protein to perform additional functions that are not related to protein folding (reviewed in Kaul et al. 2007).

Homologues for Mdj1 and Mge1 were found in humans and named Tid1 and hMge1, respectively (Supplemental Fig. 2). Human Tid1 encodes for two mitochondrial matrix-localized splice variants, one of 43 (Tid1-L) and one of 40 kDa (Tid1-S). Both variants have an N-terminal mitochondrial signal. However, they differ in their carboxyl terminus tails: Tid1-L has 33 amino acids, whereas Tid1-S has only six amino acids (Syken et al. 1999). Several lines of evidence indicate that both Tid1 isoforms are functional homologues of Mdj1: (1) they contain the characteristic J-domain of the 40-kDa heat shock protein (Hsp40) family; (2) both isoforms were co-immunoprecipitated with mortalin (Syken et al. 1999); and (3) Tid1-L and Tid1-S were both able to compensate for an Mdj1 deletion in yeast. This capacity was dependent on the presence of an intact J-domain and a mitochondrial targeting sequence (Lu et al. 2006). Tid1 isoforms were also found to be involved in extra-mitochondrial protein interactions and functions (see below). These functions include regulation of cell death, proliferation, and signal transduction (Mitra et al. 2009; Syken et al. 1999). It was shown that the two isoforms differ in their non-mitochondrial functions due to differences in their abilities to interact with cytosolic and nuclear proteins (Lu et al. 2006). Moreover, it was demonstrated that expression of the long variant increased apoptosis, while expression of the short variant suppressed apoptosis (Syken et al. 1999). Interestingly, Tid1-L was shown to be more stable in the cytosol as compared with Tid1-S, with a longer cytosolic residency time prior to mitochondrial import (Lu et al. 2006).

Molecular chaperones are key players in maintaining cellular protein homeostasis. Consistent with this role, several studies have shown that mortalin may be implicated in the progression of neurodegenerative and protein aggregation disorders, such as Parkinson and Alzheimer (Burbulla et al. 2010; De Mena et al. 2009; Deocaris et al. 2008; Deocaris et al. 2007; Koren et al. 2009; Shi et al. 2008). Moreover, when mammalian mitochondria are challenged with an imported protein with a strong tendency to form aggregates, the mitochondrial unfolded protein response (mUPR) is activated (Aldridge et al. 2007; Zhao et al. 2002). mUPR upregulated the ClpP protease and the cpn60, cpn10, and Tid1 chaperones but not mortalin.

The strong cyto-protective, even curing effects of the Hsp70–Hsp40 chaperones systems, both in the cytoplasm and in mitochondria can be attributed to their ability to act as polypeptide unfolding enzymes that can target cytotoxic misfolded and aggregated protein conformers (Sharma et al. 2009; Sharma et al. 2010). In organisms and cellular compartments that also express Hsp100/ClpB homologues, the Hsp70–Hsp40 unfoldase chaperone systems benefit

from a powerful synergic mechanism of forceful disaggregation that may act both upstream and downstream to the polypeptide unfoldase activity of Hsp70 chaperones (Glover and Lindquist 1998; Goloubinoff et al. 1999; Weibezahn et al. 2005). Thus, in the yeast cytoplasm, a large number of Hsp70–Hsp40 can collaborate with the ClpB-like (Hsp104) disaggregating co-chaperone to prevent the formation of misfolded species and even solubilize-resistant prions. Similarly, yeast mitochondria can use the unfolding abilities of Ssc1, which together with Mdj1 can collaborate with the ClpB homologue, Hsp78, at the active unfolding and disaggregation of potentially toxic misfolded protein conformers (von Janowsky et al. 2006).

Interestingly, mammalian cytoplasm contains no Hsp104 and mammalian mitochondria contain a single Hsp70 (mortalin) and no ClpB-like disaggregating co-chaperones. Notably, up to date it remained unclear whether mortalin in cooperation with Tid1 can reactivate protein aggregates. Here, we used purified components and found that mortalin and both Tid1 variants can mediate the reactivation of stable protein aggregates, implying that mortalin can serve as a unique scavenger of toxic protein conformers in human mitochondrial making it an attractive target for therapies against protein conformational diseases.

Results and discussion

Purification of mortalin and Tid1-L/Tid1-S

Upon expression in bacteria, mitochondrial Hsp70s tend to aggregate and are detected in inclusion bodies (Blamowska et al. 2010; Zhai et al. 2008). In order to obtain soluble mortalin (or Ssc1), each protein was co-expressed in bacteria together with Hep1/Zim17, which has been shown to prevent the aggregation of Ssc1 and of mortalin in mitochondria as well as upon expression in bacteria (Sichting et al. 2005; Zhai et al. 2008). Furthermore, mortalin, Ssc1 and yeast Hep1 were each cloned carrying an octa-histidine tag (His-tag), which in the case of mortalin, and Ssc1, could be cleaved by tobacco etch virus (TEV) protease (Iosefson and Azem 2010). This cloning strategy enabled the efficient removal of Hep1 on a second Ni-Agarose column following cleavage of the hsp70s His-tag by TEV. Using the purification protocol that is detailed under “Materials and methods,” we obtained purified mortalin and Ssc1 (Supplemental Fig. 3) that, in contrast to previously described protocols, are lacking the His-tag (Zhai et al. 2008; Goswami et al. 2010). The absence of Hep1 contamination in the purified protein was further confirmed by western blots with His-tagged antibodies (not shown).

Upon their expression in bacteria, both Tid1-L and Tid1-S were detected in the inclusion bodies. Thus, in the first

step, Tid1-L/S were solubilized by treatment with 3 M urea as described previously for Mdj1 (Horst et al. 1997). Final purification was carried out using a second Ni-NTA column followed by a refolding step using dialysis, as detailed under “Materials and methods.” The purity of Tid1-L is presented under Supplemental Fig. 4. Since a previous study showed that both Tid1-L and Tid1-S are equally able to complement deletion of yeast Mdj1, we initially focused on studying Tid1-L (Lu et al. 2006).

Functional cooperation between Hsp70 and DnaJ homologues as examined by single-turnover ATPase assays

As mentioned above, yeast mitochondria harbor three Hsp70 family members while human mitochondria have only one member, mortalin. Thus, one aspect of this work was to examine the ability of yeast and human Hsp70 chaperones to functionally interact with endogenous and heterologous counterparts. Because it has been extensively characterized, the closely related bacterial Hsp70 chaperone, DnaK, was also examined. We first addressed the basal ATP hydrolysis activity of the three Hsp70 chaperones using a single-turnover assay. As shown in Fig. 1, the three related Hsp70 chaperones exhibited very low ATP hydrolysis rates, of the same order of magnitude. DnaK had the highest ATP hydrolysis rate ($3.71 \cdot 10^{-4} \text{ s}^{-1}$), while the basal ATPase activity of mortalin and Ssc1 was slightly lower ($2.04 \cdot 10^{-4}$ and $2.56 \cdot 10^{-4} \text{ s}^{-1}$, respectively).

A functional interaction between Mdj1 and the chaperones DnaK, mortalin, and Ssc1 is clearly revealed (Fig. 1) by the fact that the ATPase activity of all Hsp70s is significantly enhanced by the co-chaperone (399.0-, 22.3- and 4.8-fold, respectively). Tid1-L was also able to functionally interact with the three Hsp70 chaperones. However, the ATPase of DnaK and mortalin, was enhanced to a much less degree in the presence of Tid1-L than in the presence of Mdj1 (9.6 and 8.7, respectively). The increase in ATPase rate that was obtained with Tid1-L was specific since no enhancement was observed using the inactive Tid1-L mutant H121Q (Supplemental Fig. 5). Thus, we demonstrated that the bacterial, yeast and human Hsp70s are able to functionally interact with both Mdj1 and Tid1-L, results which reflect the evolutionary conservation of this interaction.

DnaK, Ssc1, and mortalin are able to reactivate protein aggregates when assisted by Mdj1

Previous studies have shown that DnaK and Ssc1 can reactivate stable protein aggregates when assisted by bacterial DnaJ and GrpE co-chaperones (De Los Rios et al. 2006; Mogk et al. 1999). However, the ability of mortalin to reactivate preformed protein aggregates has not

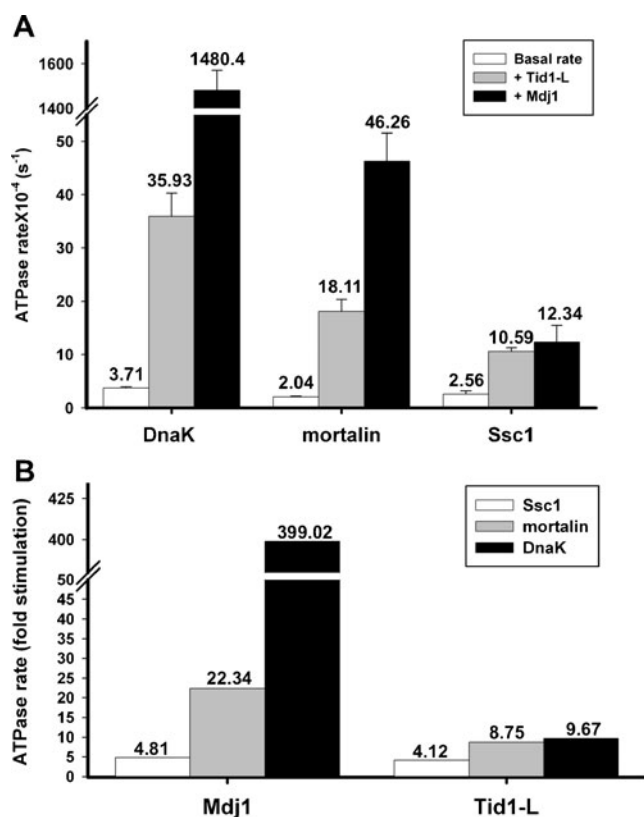


Fig. 1 Stimulation of Hsp70 ATPase activity by Mdj1 or Tid1-L. **a** The indicated Hsp70/ATP complexes were incubated in the presence or absence of either Mdj1 or Tid1-L. The indicated turnover values, expressed as the rate of ATP hydrolysis per second, were obtained from the slope of the equation describing the linear portion of the reaction. **b** The ATPase activity is presented as fold stimulation of Hsp70 by either Mdj1 or Tid1-L

been demonstrated so far. The time-dependent reactivation of G6PDH by DnaK, Ssc1 and mortalin was first examined in the presence of yeast Mdj1 and Mge1. As shown in Fig. 2, when examined with Mdj1, DnaK, and mortalin were both able to efficiently reactivate G6PDH aggregates, with DnaK showing slightly faster initial rates of activity ($7.3 \cdot 10^{-4}$ and $5.7 \cdot 10^{-4} \text{ min}^{-1}$, respectively). Interestingly, mortalin and DnaK, when assisted by yeast Mdj1, were more efficient at reactivating preformed G6PDH aggregates than Ssc1, the yeast partner. When we examined the dependence of Ssc1 reactivation activity on Mdj1 concentration, we found that it reached a maximum at $0.5 \mu\text{M}$ of the J-protein (Fig. 2, inset). Upon further increase in Mdj1 concentration, however, the reactivation activity decreased, similar to what was shown previously for DnaK and DnaJ (Laufen et al. 1999). In contrast to Ssc1 activity, DnaK and mortalin activity did not decrease in the presence of the higher Mdj1 concentrations. Still, at the optimal Mdj1 concentration examined in this experiment, the maximal yield of reactivated G6PDH in the presence of Ssc1 reached only 30%.

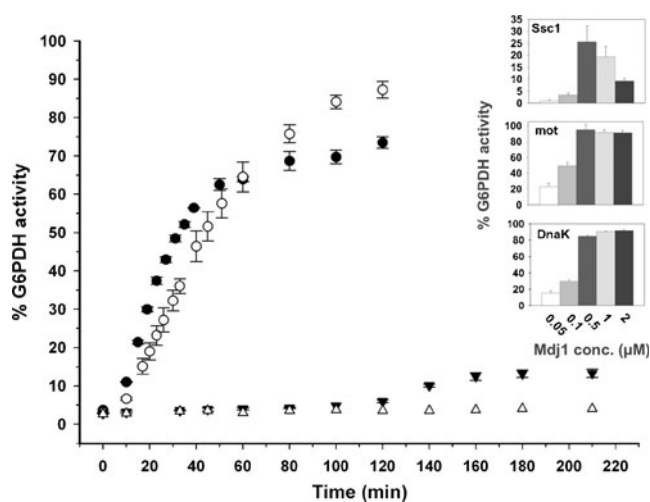


Fig. 2 Time-dependent reactivation of stable G6PDH aggregates by DnaK, Ssc1, and mortalin. Stable G6PDH aggregates were reactivated at 30°C in the presence of $16 \mu\text{M}$ of either DnaK (filled circles), Ssc1 (filled inverted triangles) or mortalin (empty circles), and yeast co-chaperones Mdj1 ($2 \mu\text{M}$) and Mge1 ($1 \mu\text{M}$). The spontaneous refolding of the aggregates in the presence of the reaction buffer is also shown (empty triangles). The insets describe the G6PDH activity after reactivation in the presence of $16 \mu\text{M}$ of either DnaK, Ssc1 or mortalin, the indicated concentrations of Mdj1, and $1 \mu\text{M}$ of Mge1. G6PDH activity was measured after 230 min at 30°C . Background activity was subtracted

Tid1-L can assist DnaK and mortalin, but not Ssc1, in mediating the ATP-dependent reactivation of G6PDH aggregates

The above result demonstrated for the first time that mortalin can reactivate G6PDH aggregates, albeit only in the presence of yeast Mdj1. We next examined whether mortalin's partner, Tid1-L, is also able to assist the three chaperones in the reactivation of preformed G6PDH aggregates. The specificity of the reactivation activity was confirmed by examining the inactive mutant of Tid1-L, H121Q. In addition, mortalin-V482F (Iosefson and Azem 2010), a mutant exhibiting a defective interaction with client proteins, failed to reactivate G6PDH preformed aggregates (Supplemental Fig. 6). As shown in Fig. 3, Tid1-L was efficiently able to assist both DnaK and mortalin in mediating the reactivation of aggregates. Interestingly, mortalin was more efficient at reactivating G6PDH when it was assisted by Mdj1 than when assisted by its own endogenous co-chaperone, Tid1-L. Noticeably, Ssc1 was much less efficient in its ability to reactivate protein aggregates even when assisted with its endogenous co-chaperone. Moreover, Ssc1 was unable to reactivate preformed G6PDH aggregates at all in combination with Tid1-L.

In summary, we found that among the three chaperones, Ssc1 acted as a poor reactivation machinery as compared

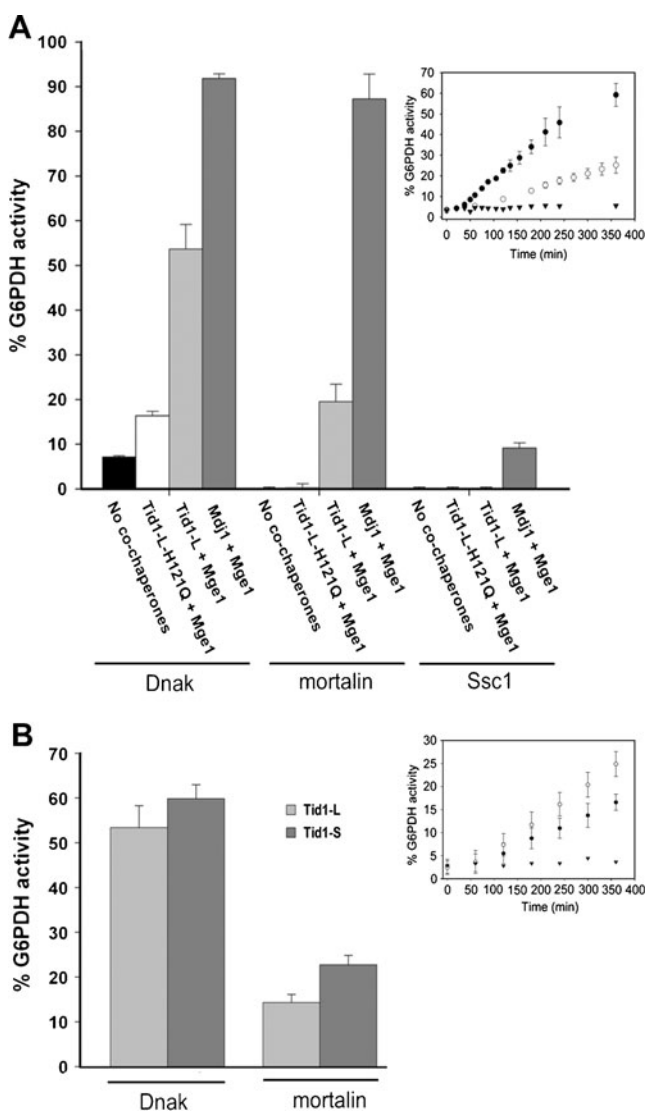


Fig. 3 Yield of G6PDH refolding (after 6 h) obtained by DnaK, Ssc1, and mortalin. **a** Stable G6PDH aggregates were reactivated at 30°C in the presence of DnaK, Ssc1, or mortalin (16 μ M), either alone or assisted by the indicated J-protein (2 μ M) and Mge1 (1 μ M). The presented values are averages of at least three different experiments with the background activity subtracted. The *inset* describes the time-dependent G6PDH activity in the presence of 16 μ M DnaK (filled circles) or mortalin (empty circles) assisted by 2 μ M Tid1-L and 1 μ M Mge1. The spontaneous refolding of the aggregates in the presence of the reaction buffer is also shown (filled inverted triangles). **b** Same conditions as in (a), except that 1 μ M of Tid1-L or Tid1-S was used. The *inset* describes the time-dependent G6PDH activity in the presence of 16 μ M mortalin assisted by either 1 μ M of Tid1-L (filled circles) or 1 μ M of Tid1-S (empty circles) and 1 μ M Mge1

with DnaK and mortalin, whereas, Tid1-L was a less efficient co-chaperone of the reactivation reaction than yeast Mdj1.

As mentioned above, in addition to Tid1-L human cells express also another spliced form, Tid1-S. It was shown that Tid1-L is much more stable in the cytosol than Tid1-S, while both were equally stable inside the mitochondria (Lu

et al. 2006). Thus, it was very interesting to examine whether Tid1-S and Tid1-L exhibit different reactivation activities. To this end, we also examined the ability of Tid1-L and Tid1-S to reactivate preformed G6PDH aggregates. Interestingly, Tid1-S was more active in the reactivation of aggregates than Tid1-L, with rates of reactivation $3.12 \cdot 10^{-5}$ and $1.56 \cdot 10^{-5} \text{ min}^{-1}$, respectively (Table 2 and Fig. 3b).

The efficiency of disaggregation is determined by chaperone/co-chaperone combination

Finally, we examined the efficiency of the disaggregation activity exerted by the three chaperones when assisted by either Mdj1 or Tid1-L and analyzed the steady-state ATPase rate of Hsp70s under the same conditions as the disaggregation reaction. As presented in Table 1, DnaK and mortalin worked with a nearly similar efficiency when Mdj1 was included in the disaggregation reaction, hydrolyzing almost $\sim 3,150$ molecules of ATP for each reactivated G6PDH molecule. Interestingly, DnaK and mortalin exhibited lower efficiency in the presence of Tid1-L, hydrolyzing 12,800 and 151,282 molecules of ATP for each refolded G6PDH molecule, respectively. Since mortalin exhibited similar steady-state ATP hydrolysis rates in the presence of both Tid1-L and Tid1-S, the immediate outcome should be that mortalin functions more efficiently in the presence of Tid1-S than Tid1-L, in terms of number of ATP molecules hydrolyzed per reactivated G6PDH molecules (Table 2).

Concluding remarks

Molecular chaperones serve as a first line of defense against uncontrolled unfolding and aggregation of proteins (Broadley and Hartl 2009). Mitochondria serve as the major site of ATP production, a process which involves electron transfer and free radical production. Thus, its proteins face continuous

Table 1 Disaggregation efficiencies of the studied chaperones

	J-protein	DnaK	Mortalin	Ssc1
ATPase (min^{-1})	Mdj1	2.3 \pm 0.2	1.82 \pm 0.1	1.58 \pm 0.08
	Tid1-L	1.6 \pm 0.17	2.36 \pm 0.24	1.77
G6PDH disaggregation (min^{-1})	Mdj1	7.3 $\cdot 10^{-4}$ \pm 9 $\cdot 10^{-5}$	5.7 $\cdot 10^{-4}$ \pm 9 $\cdot 10^{-5}$	6.25 $\cdot 10^{-5}$
	Tid1-L	1.25 $\cdot 10^{-4}$ \pm 2.2 $\cdot 10^{-5}$	1.56 $\cdot 10^{-5}$	NA
nmol ATP/nmol G6PDH refolded	Mdj1	3,150	3,193	25,280
	Tid1-L	12,800	151,282	–

Turnover numbers of steady-state ATPase and G6PDH disaggregation were determined as described under “Materials and methods”; 16 μ M of the indicated Hsp70 proteins, 2 μ M of the relevant J-domain protein, and 1 μ M of Mge1 were used

NA not active

Table 2 Comparison of disaggregation efficiencies between Tid1 splice variants

	J-protein	DnaK	Mortalin
ATPase (min^{-1})	Tid1-L	1.33±0.085	1.99±0.16
	Tid1-S	2.06±0.057	1.93±0.14
G6PDH disaggregation (min^{-1})	Tid1-L	7.29·10 ⁻⁵ ±9·10 ⁻⁶	1.56·10 ⁻⁵
	Tid1-S	9.3·10 ⁻⁵	3.12·10 ⁻⁵
nmol ATP/nmol G6PDH refolded	Tid1-L	18,244	127,564
	Tid1-S	22,150	61,858

Turnover numbers of steady-state ATPase and G6PDH disaggregation were determined as described under “Materials and methods”; 16 μM of the indicated Hsp70 proteins, 1 μM of the relevant Tid1 construct, and 1 μM of Mge1 were used

stress, which if not countered, may lead to protein aggregation and cell death (de Castro et al. 2010; Pedersen et al. 2003; Schapira 1998; Xie et al. 2010; Vijayvergiya et al. 2005). Expression of aggregation-prone mutant form of ornithine transcarbamylase in the mitochondrial matrix leads to the induction of the mUPR (Zhao et al. 2002). Interestingly, the mammalian homologue of Tid1, but not mortalin, was among the proteins that were induced upon mUPR (Zhao et al. 2002).

While an interaction between Tid1 and mortalin has been demonstrated previously (Goswami et al. 2010), the ability of these proteins to cooperate in reactivating preformed protein aggregates has not been demonstrated so far. In situations of folding malfunction, reactivation of protein aggregates is clearly a critical function for continued viability of the cell.

Using G6PDH as a model substrate, we showed for the first time that mortalin and Tid1 splice variants, can mediate disaggregation of preformed G6PDH protein aggregates and refolding of the enzyme to a functional state. Interestingly, in vitro, Tid1-L was more active with the bacterial Hsp70, DnaK, than with its endogenous chaperone mortalin, and it was unable to assist Ssc1 in reactivating protein aggregates in an ATP-dependent manner. This probably reflects the fact that DnaK is much stronger ATPase than mortalin. Nevertheless, in terms of efficiency, both DnaK and mortalin consumed similar amounts of ATP per refolded G6PDH when examined with yeast Mdj1 (Table 1).

Of the two spliced forms found in human cells Tid1-S was more efficient in mediating the reactivation of protein aggregates than Tid1-L. This is in accordance with previous study, in which Tid1-S was more efficient in its ability to prevent aggregation of denatured rhodanese than Tid1-L (Goswami et al. 2010). More work is required in order to show whether this observation is related to the fact that Tid1-L is more stable in the cytosol than Tid1-S (Lu et al. 2006).

A recent, single-molecule analysis of DnaK and Ssc1 showed that, despite their evolutionary conservation, these proteins exhibited different conformational changes upon

their interaction with ADP and their co-chaperones (Mapa et al. 2010). These differences were suggested to help the chaperones to meet specific functions in different organisms and cellular compartments (Mapa et al. 2010). It is tempting to speculate that the conformational differences between the various chaperones may also be reflected by distinct abilities to reactivate misfolded protein aggregates.

Because mammalian mitochondria contain a single Hsp70 (mortalin), a single Hsp40 (Tid1) and no ClpB-like disaggregating co-chaperones, mortalin remains the only known mitochondrial chaperone that, together with Tid1, could carry the active detoxification of harmful protein aggregates by unfolding them and, thus, provide stressed human mitochondria with a precious protective mechanism against apoptosis. It is tempting to speculate that non-toxic drugs that could induce the accumulation of mortalin and Tid1 could also reduce toxic protein aggregation in mitochondria and thus block apoptotic signals in inflammatory protein misfolding diseases and aging. Conversely, drugs directly blocking mortalin and Tid1 would favor apoptosis in cancer cell.

Materials and methods

Cloning of mortalin or Ssc1 and yeast Hep1 into the pETDuet co-expression vector

The nucleotide sequence encoding residues 51–679 of mature mortalin was generated by PCR from a human cDNA genomic library and cloned between the BamHI (forward primer-I; Supplemental Table 1) and NotI (backward primer-II) restriction sites. The nucleotide sequence encoding amino acid residues 49–171 of mature *S. cerevisiae* Hep1 was cloned between the NdeI (forward primer-III) and XhoI (backward primer-IV) restriction sites. The two PCR products were digested with suitable restriction enzymes and ligated into a modified pETDuet vector (Iosefson and Azem 2010). The resulting plasmid co-expresses mortalin and yeast Hep1. Both proteins were engineered to contain a His-tag at their N terminus, however; only mortalin's His-tag can be cleaved by the TEV protease. The pETDuet plasmid was transformed into *E. coli* which served as a host for co-expression of mortalin and Hep1. Similar strategy was used to create co-expression vectors for Ssc1 and Hep1. A mortalin-V482F mutant was generated using the pETDuet plasmid as a template for mutagenesis according to the *i-Pfu* DNA polymerase protocol (Intron).

Purification of mortalin and Ssc1

The *E. coli* BL-21 Tuner strain carrying the pETDuet plasmid was grown for 3–4 h at 37°C in LB media,

containing 200 µg/ml ampicillin and 34 µg/ml chloramphenicol. Protein expression was induced with 1 mM of IPTG for 16 h at 16°C. Next, cells were harvested and resuspended (1:10, w/v) in buffer A (20 mM MES, pH 6.6, and 5% glycerol) containing 45 U/ml of DNase, 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mg/ml lysozyme, and one tablet of complete EDTA-free protease inhibitor cocktail (Roche). After incubation for 15 min at 4°C, bacteria were homogenized and disrupted using a micro-fluidizer (Microfluidic). The soluble fraction was loaded on an anion-exchange column (RESOURCE Q, ~30 ml bead volume, Amersham) pre-equilibrated with buffer A. The column was washed with ~2 L of buffer A until the majority of Hep1 was removed. Fractions were eluted with a linear gradient (0–50%, 90 ml) of buffer B (buffer A containing 1 M NaCl). Fractions enriched with the desired protein were pooled, diluted 1:5 with buffer C (50 mM Tris-HCl, pH 7.7, 200 mM NaCl, 5% glycerol and 20 mM imidazole) and loaded on a Ni-NTA column (~15 ml bead volume) pre-equilibrated with buffer C. The column was washed with buffer C until a stable baseline was observed. Fractions were eluted with a linear gradient (0–50%, 90 ml) of buffer D (buffer C containing 1 M imidazole). Fractions enriched with the protein were pooled and treated with TEV protease (TEV/protein 1:25, w/w) overnight at 4°C, in a tubular dialysis membrane (12–14 kDa MWCO) against 4 L of buffer E (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5% glycerol, and 20 mM imidazole). After TEV digestion, the protein was loaded onto a second nickel column to remove: (1) TEV protease and the residual Hep1, both containing an uncleavable His-tag; (2) the uncleaved protein of interest that still contained the His-tag; and (3) other nonspecifically bound proteins. Relevant fractions were concentrated using protein concentrators (M_w cutoff of 30,000, Millipore) and desalted using PD10 columns (Pharmacia) with buffer F (20 mM Na-HEPES, pH 7.4, 200 mM NaCl, and 5% glycerol). The protein was divided into aliquots, flash-frozen in liquid N₂ and stored at –80°C until use. All purification procedures were carried out at 4°C. We confirmed the absence of residual Hep1 protein in the purified mortalin preparations using western blotting with anti-His-tag antibodies. Mortalin-V482F was purified according to the same protocol.

Cloning of Tid1-L and Tid1-S into the pET21d expression vector

The nucleotide sequence encoding mature Tid1-L (residues 66–480) or Tid1-S (residues 66–453) were generated by PCR from a human cDNA genomic library with BamHI (forward primer-VII) and XhoI (backward primers-VIII and XV, respectively) restriction sites. The PCR product was

cloned into the pGEM-T Easy vector, transformed into XL1-Blue *E. coli* competent cells and plated onto LB-plates supplemented with 200 µg/ml ampicillin. Plasmids were isolated from the positive colonies and sequenced to rule out PCR-induced sequence errors. The construct was digested with BamHI and XhoI and ligated into a modified pET21d (+) vector. The protein was engineered to contain a His-tag at its N terminus that is cleavable by TEV protease. However, in the function experiments described in this work, the tag was not removed. The pET21d (+) plasmid was transformed into BL-21 *E. coli* competent cells for subsequent expression. A Tid1-L-H121Q mutant was generated using the pET21d plasmid as a template for mutagenesis according to the *i-Pfu* DNA polymerase protocol (Intron).

Purification of Tid1 proteins

E. coli BL-21 tuner strain, carrying the pET21d (+) plasmid with the relevant Tid1 construct, was grown for 3–4 h at 37°C in LB media, containing 200 µg/ml ampicillin and 34 µg/ml chloramphenicol. Protein expression was induced with 1 mM of IPTG. After 4–5 h, at 16°C, cells were harvested and suspended (1:10, w/v) in buffer A (20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 10% glycerol, 1% Triton X-100, 0.05% Brij-58, and 2 mM β-mercaptoethanol) containing 45 U/ml of DNase, 2 mM PMSF, 0.1 mg/ml lysozyme, and one tablet of complete EDTA-free protease inhibitor cocktail. After incubation for 15 min at 4°C, bacteria were homogenized and disrupted using a micro-fluidizer and the sample was centrifuged at 14,000 rpm for 1 h at 4°C. Tid1 was found in the pellet fraction (inclusion bodies) together with a small number of additional proteins. Pure inclusion bodies were obtained by three sequential cycles of pellet resuspension in buffer A and centrifugation at 14,000 rpm for 30 min. In a preliminary experiment, we found that 3 M urea rescues most of Tid1-L from the inclusion bodies. Therefore, in the next step the inclusion bodies were resuspended in buffer B (buffer A containing 3 M urea and 5 mM imidazole) and incubated for 30 min on an end-over-end rotator. The insoluble material was removed by centrifugation for 30 min at 14,000 rpm. The soluble fraction was incubated for 1 h at 4°C with Ni-NTA beads that had been pre-equilibrated with buffer B (~2 ml bead volume), on an end-over-end rotator. The beads were centrifuged for 2 min at 2,000 rpm and supernatant containing unbound proteins was removed. Next, the beads were washed twice with buffer B and twice more with buffer C (buffer B lacking Triton X-100). Bound proteins were eluted by incubating the Ni-NTA beads for 30 min at 4°C with buffer D (buffer C containing 300 mM imidazole). The eluted fraction, containing Tid1, was separated from the beads by centrifugation for 2 min at 2,000 rpm. The

urea was removed from the Tid1 solution by overnight dialysis in a tubular dialysis membrane against 2 L of buffer E (20 mM Tris, pH 7.4, 0.5 M NaCl, 10% glycerol, 0.05% Brij-35, 2 μ M β -mercaptoethanol, and 5 mM imidazole). A significant amount of protein aggregated after the urea removal. The insoluble material was removed by centrifugation at 14,000 rpm for 1 h. The soluble fraction (~50 ml) was purified by a second incubation with Ni-NTA beads (~1 ml bead volume), in a batch procedure. The following few modifications were made compared with the first Ni-NTA purification: (1) the Ni-NTA beads were pre-equilibrated with buffer E; (2) after protein binding, the beads were washed four times with buffer E; (3) the proteins were eluted with 2.5 ml buffer F (buffer E containing 300 mM imidazole). The eluted fraction was desalted (PD-10 columns) in 3.5 ml of buffer G (buffer F without imidazole), divided into aliquots, and flash-frozen in liquid N₂. All purification procedures were carried out at 4°C.

Cloning and purification of DnaK

DnaK was cloned into a modified pET21d that contained a TEV protease site between the protein and the His-tag. The engineered plasmid expresses the protein containing an octa-His-tag at its N terminus that can be removed after treatment with TEV protease. In general, DnaK was purified in four steps: (1) purification on Ni-NTA agarose beads, (2) cleavage of the tag with TEV protease, (3) second Ni-agarose to remove uncut protein and the His-tagged TEV protease, and (4) gel filtration using a Superdex 200 column.

Protein concentration determination

The concentrations of Ssc1, mortalin, DnaK, and Mge1 were determined using the bicinchoninic acid protein assay with BSA as a standard. The concentrations of Tid1 constructs and Mdj1 were obtained through their absorbance at 280 nm and using the predicted molar extinction coefficient of the proteins. These values were determined using the ProtParam ExPASy proteomic tool (<http://www.expasy.org/tools/protparam.html>). The molar extinction coefficients (M⁻¹ cm⁻¹) at 280 nm were: 29,380 for Mdj1 and 31,330 for Tid1.

Formation of the Hsp70-ATP complex and single-turnover analysis

Mortalin, DnaK, or Ssc1 were incubated with 10 μ Ci [α -³²P] ATP (3,000 Ci/mmol, Izotop) in buffer A (50 mM Tris acetate, 100 mM potassium acetate, 10 mM magnesium acetate, pH 7.4, and 2 mM DTT) containing 25- μ M cold ATP. Mortalin and Ssc1 were incubated at room

temperature for 5 and 2.5 min, respectively, while DnaK was incubated for only 2 s at 4°C. Then, the complex was isolated by size exclusion chromatography column (PD-10, Amersham) at 4°C, pre-equilibrated with buffer A; ~250 μ l fractions were collected. The radioactivity of the fractions was monitored via a β -counter (PerkinElmer). The first peak of radioactivity that contained the Hsp70/ATP complex was collected, divided into aliquots, and flash-frozen in liquid N₂.

For single-turnover experiments, the complex was quickly thawed and 10–12 μ l was added to an equal amount of buffer B (20 mM Na-HEPES, pH 7.4, 100 mM KCl, 100 mM NaCl, 10 mM MgCl₂, and 2 mM DTT) containing various factors and incubated at 30°C. At the indicated time points, 3 μ l of the reaction mixture was withdrawn and mixed with 3 μ l buffer C (250 mM EDTA, pH 8, and 25 mM ATP) to stop the reaction. The mixture was loaded on a TLC membrane and developed for 30 min by using 0.5 M sodium phosphate, pH 3.5, as the mobile phase. The membranes were dried and exposed in BAS CASSETTE 2040 (FUJIX) overnight. The ADP and ATP bands were detected using phosphor imager (FLA-5100, FUJI) and quantified by the Image Gauge (version 4) program (FUJIFILM).

In vitro refolding of heat-denatured G6PDH

The refolding of heat-denatured glucose-6-phosphate dehydrogenase (G6PDH) was carried out as described previously (Diamant et al. 2000) with minor modifications. G6PDH (0.85 μ M) from *Leuconostoc mesenteroides* (Sigma) was incubated for ~15 min at 52°C in buffer A (100 mM Tris, pH 7.4, 100 mM KCl, 10 mM MgCl₂, and 10 mM DTT). The remaining activity after the denaturation step was <2%. The stable aggregates were incubated for 10 min at 4°C before adding the ATP regenerating mixture (0.2 M glycine betaine monohydrate, 10 μ g/ml pyruvate kinase, 5 mM phosphoenol pyruvate, and 4 mM ATP). Then the chaperone system was added (16 μ M of the indicated Hsp70 proteins, 2 or 1 μ M of the relevant J-domain protein, and 1 μ M of the nucleotide exchange factor). The final concentrations of G6PDH and ATP after these dilutions were 0.64 μ M and 3 mM, respectively. The entire mixture was incubated at 30°C, and G6PDH enzymatic activity was measured at room temperature as previously described (Hansen and Gafni 1993).

Steady-state ATPase measurements

The same conditions used in G6PDH refolding assays were applied to the steady-state ATPase measurements, but in the absence pyruvate kinase; 16 μ M of the various Hsp70s, 2 or 1 μ M of the relevant J-domain protein, and 1 μ M Mge1 were incubated in buffer A (100 mM Tris-HCl, pH 7.4, 100 mM

KCl, 10 mM MgCl₂, 10 mM DTT, 0.2 M glycine betaine monohydrate, 5 mM phosphoenol pyruvate, and 3 mM ATP) in the presence of 0.64 μM G6PDH and 0.01 μCi [γ -³²P] ATP (6,000 Ci/mmol, Izotop) at 30°C. Samples from the required time points were taken and separated on TLC as described for single-turnover experiments.

Miscellaneous

Mdj1 and yeast Mge1 were cloned and purified as described previously (Deloche et al. 1997; Horst et al. 1997).

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