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Role of the mitochondrial DnaJ homologue, Mdj1p, in the prevention of heat-induced protein aggregation

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Abstract The role of the mitochondrial Hsp70 system in the prevention of heat-induced protein aggregation was studied in isolated mitochondria from *Saccharomyces cerevisiae*. Firefly luciferase was employed as a thermolabile tester protein. After shift to 40°C a transient increase of mt-Hsp70/luciferase complex was observed, which required functional Mdj1p and Mge1p, the mitochondrial homologues of DnaJ and GrpE. The kinetics of luciferase aggregation, however, were not influenced by mutations in either mt-Hsp70 or Mge1p. Only the absence of Mdj1p led to enhanced protein aggregation. Thus, a central role in the transient protection against heat stress is attributed to this mitochondrial DnaJ homologue.

Key words: Mitochondrial Hsp70 system; Mdj1p; Firefly luciferase; Heat-induced protein aggregation; *Saccharomyces cerevisiae*

1. Introduction

Many proteins undergo denaturation under physiological stress such as elevated temperature [1,2]. Various components and pathways exist which protect cells against heat stress by reducing protein aggregation and/or by promoting disaggregation of aggregates. Furthermore there are mechanisms which facilitate either protein refolding or proteolytic breakdown of irreversibly damaged proteins. These protective mechanisms are mediated by molecular chaperones [3–7].

Protein folding and prevention of protein aggregation has been extensively studied with purified components in vitro. In only a few cases these processes have been analyzed in intact cells. In *Escherichia coli*, the two major Hsps, DnaK and GroEL (the prokaryotic Hsp70 and Hsp60 homologues), have been demonstrated to be involved in the reactivation of heat denatured firefly luciferase [5]. The two cohort chaperones of DnaK, DnaJ and GrpE, are required to allow reactivation of thermally inactivated luciferase [5]. From in vitro studies it became clear that DnaJ stimulates the ATPase activity of DnaK and targets DnaK to substrate proteins [8–10]. GrpE acts as a nucleotide exchange factor [10]. Yet little is known about the mechanisms by which Hsp70 and its cohorts fulfil their protective role in vivo during heat stress by reducing aggregation.

Protection against heat-induced protein damage can be monitored in isolated mitochondria which constitute an organellar in vivo system. Mitochondria contain the major heat shock proteins, Hsp70 and Hsp60 (mt-Hsp70 and mt-Hsp60) (see [11] for review). More recently the cohort chaperones of mt-Hsp70, mitochondrial DnaJ, (Mdj1p) and mitochondrial GrpE (Mge1p) have been identified and characterized [12–15]. Mt-Hsp70 and Mge1p have been recognized to constitute essential components of the mitochondrial protein import machinery [14–17]. In contrast, Mdj1p does not play an indispensible role in protein import. Rather, the protein is involved in the folding of newly imported proteins in the mitochondrial matrix [12]. Both mt-Hsp70 and Mge1p participate in this process, and mutations in Mge1p result in enhanced protein aggregation [16,17]. A sequential action of mt-Hsp70 and mt-Hsp60 after import has been suggested: imported precursor proteins are passed from mt-Hsp70 to mt-Hsp60 with the latter mediating final folding steps [18,19]. Both mt-Hsp60 and Mdj1p confer limited protection against heat-induced protein denaturation as matrix-located thermosensitive tester proteins are prone to unfolding when functional mt-Hsp60 or Mdj1p are absent [4,12,18].

In this study we have analyzed heat-induced luciferase aggregation in mitochondria in which heat shock proteins were absent or their functions were compromized by mutations. The results suggest that mt-Hsp70 together with Mdj1p and Mge1p is involved in the protection against heat-induced protein aggregation. However, only Mdj1p plays an essential role in this process.

2. Materials and methods

2.1. Yeast strains and isolation of mitochondria

Saccharomyces cerevisiae strain W303 served as the wild-type control strain for the analysis of the Mge1p mutants, the Δmdj1 disruption strain and rho° control cells. For description of the strain constructions see references [12,17]. Isogenic wild-type for the mt-Hsp70 mutants was strain PK82 [20]. Strain construction of the mt-Hsp70 mutants is detailed in reference [16]. For isolation of mitochondria [22], strains were cultivated at 24°C in lactate medium as described [21] with the modification that 1% galactose was added to the medium instead of glucose.

2.2. Determination of luciferase aggregation

^{35}S-radiolabeled pSu9-luciferase was synthesized in reticulocyte lysate and import into mitochondria was carried out as described [12].

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Abbreviations: E. coli, Escherichia coli; Hsp, heat shock protein; mt-Hsp60, mitochondrial heat shock protein of 60 kDa; mt-Hsp70, mitochondrial heat shock protein of 70 kDa; PMSF, phenyl-methylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; *S. cerevisiae, Saccharomyces cerevisiae.*

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Luciferase was imported into 350 μg mitochondria in 700 μl import buffer containing an ATP-regenerating system for 25 min at 25°C. Import was stopped by the addition of 1 μg/ml valinomycin. Mitochondria were reisolated by centrifugation, washed in 800 μl SMKCl (250 mM sucrose, 10 mM MOPS/KOH pH 7.2, 80 mM KCl) and resuspended in 350 μl SMKCl. The mitochondria were then incubated for 30 min on ice with 200 μg/ml proteinase K to remove non-imported luciferase. Proteinase K was inhibited by the addition of 1 mM PMSF and 15 min incubation on ice before mitochondria were collected by centrifugation. Mitochondria were resuspended in 350 μl import buffer containing an ATP-regenerating system and split into 40 μl aliquots. The aliquots were then transferred to 40°C. After various incubation periods at 40°C heat exposure was stopped by the addition of five volumes ice-cold SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS/KOH pH 7.2). The mitochondria were then collected by centrifugation and resuspended at a protein concentration of 0.5 mg/ml in SEM buffer containing 0.5% Triton X-100 and 1 mM PMSF and left for 10 min on ice. The soluble fraction was separated from the insoluble fraction by centrifugation at 16,000 × *g* for 10 min. Protein in the supernatant was precipitated by trichloroacetic acid before application to SDS-PAGE. The pellet was directly analyzed by SDS-PAGE. After fluorography the percentage of aggregated material was assessed by laser densitometry.

2.3. Co-immunoprecipitation and immunoblotting

After shift of the mitochondria to 40°C aliquots corresponding to 130 μg mitochondrial protein were removed and lysed as described [17]. Co-immunoprecipitation of luciferase with antibodies against mt-Hsp70 coupled to protein A-Sepharose was carried out as described [17].

3. Results

3.1. Association of luciferase with mt-Hsp70 at elevated temperature and role of Mge1p and Mdj1p

Firefly luciferase was used as a thermolabile tester protein to study the function of mt-Hsp70 and its co-chaperones in the prevention of heat-induced protein aggregation. Luciferase fused to the mitochondrial presequence of subunit 9 of the F_0-ATPase from *Neurospora crassa* was synthesized in vitro in the presence of [^{35}S]methionine and imported into isolated

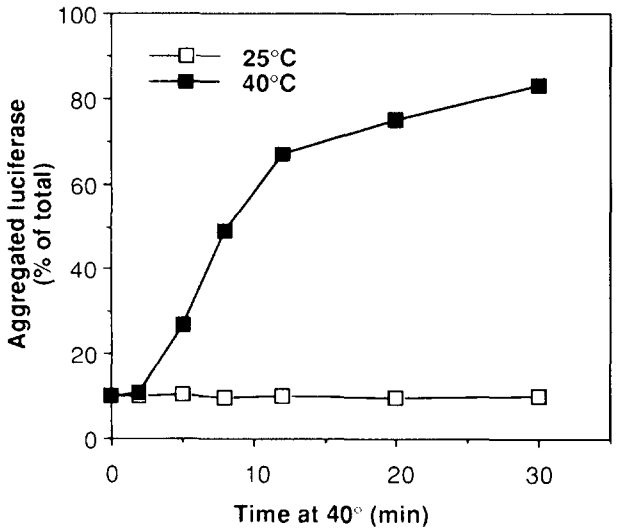

Fig. 1. Firefly luciferase imported into wild-type mitochondria aggregates at 40°C. After import of radioactively labeled luciferase, mitochondria were shifted to 40°C for the time intervals indicated (see section 2). The amount of aggregated luciferase was assessed as described in section 2. The amount of total imported species was set to 100%. The aggregated material was plotted as the percentage of total species present in the matrix.

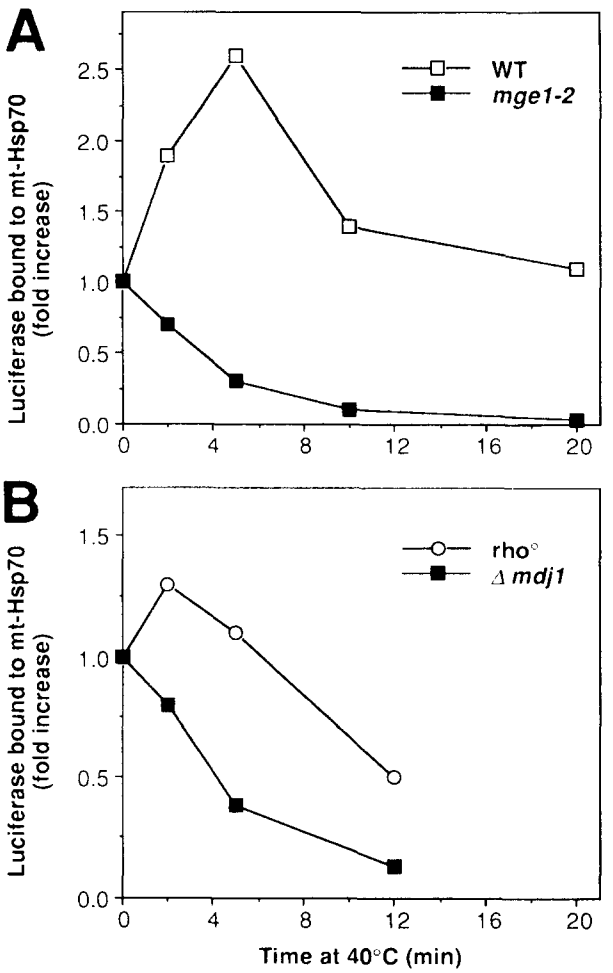

Fig. 2. Transient association of mt-Hsp70 with luciferase at 40°C depends on the presence of functional Mge1p and Mdj1p. (A) Luciferase was imported into mitochondria from wild-type strain W303 and strain W303 harboring mutant Mge1-2p [17]. Following shift to 40°C samples were withdrawn for subsequent co-immunoprecipitation. Co-immunoprecipitated luciferase was determined as percentage of total soluble luciferase for the various time points. The percentage of co-immunoprecipiated luciferase at time point zero of the heat exposure (before shift to 40°C) was set to 1. (B) Luciferase was imported into mitochondria from the *Δmdj1* strain and those from the isogenic rho° control cells. Co-immunoprecipitations and corrections were carried out as described above for panel A.

mitochondria at 25°C. After import and proteinase K digestion of non-imported material, mitochondria were either shifted to 40°C for different time periods or maintained at 25°C. Luciferase aggregated when wild-type mitochondria were shifted to 40°C. No aggregation was observed when the mitochondria were kept at 25°C (Fig. 1). The data demonstrate that firefly luciferase imported into mitochondria is prone to rapid, heat-induced aggregation.

Association of mt-Hsp70 with luciferase was studied by co-immunoprecipitation with antibodies against mt-Hsp70. A certain amount of complex (8–10% of total) was present at zero time probably because not all of the polypeptides had been released from mt-Hsp70 after import. Mitochondria were then incubated at 40°C for different time periods. With mitochondria from wild-type a transient increase of mt-Hsp70/luciferase

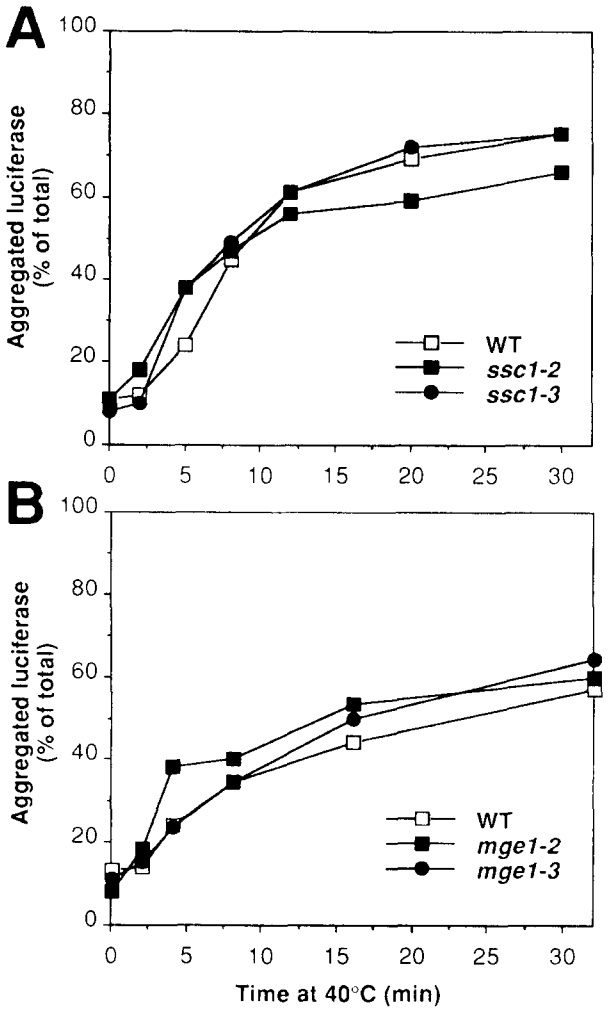

Fig. 3. Mutations in mt-Hsp70 and Mge1p do not affect the kinetics of luciferase aggregation. (A) Mitochondria with mutations in mt-Hsp70 (*ssc1-2* and *ssc1-3*) and mitochondria from the isogenic wild-type. The amount of aggregated material was plotted as the percentage of total species present in the matrix (see legend to Fig. 1). (B) Mitochondria with mutations in Mge1p and the corresponding wild-type mitochondria.

complex was detected early after shift to elevated temperature (Fig. 2A). The mt-Hsp70-associated material then decreased with time. This decline probably reflects substrate molecules which were released from mt-Hsp70 and were unable to reassociate with mt-Hsp70, since they had already undergone aggregation. Association of luciferase with mt-Hsp70 was also investigated with mitochondria containing a temperature-sensitive mutant form of Mge1p, Mge1-2p [17]. In this case transient increase of mt-hsp70/luciferase complex was not observed, rather a decrease after shift to 40°C was detected (Fig. 2A). This decrease is apparently due to a release of newly imported polypeptides which were still associated with mt-Hsp70 while being translocated into the matrix. Since the mutant phenotype of Mge1-2p is induced at 40°C [17] we conclude that Mge1p function is required for transient association of luciferase with mt-Hsp70.

Association of mt-Hsp70 with luciferase was investigated in mitochondria in which Mdj1p was absent due to disruption of the *MDJ1* gene. Disruption of *MDJ1* leads to the loss of mitochondrial DNA, i.e. a rho° state of the cells [12]. To exclude secondary effects resulting from the rho° state in *Δmdj1* mitochondria, association of luciferase with mt-Hsp70 was also analyzed in mitochondria from the isogenic rho° strain possessing Mdj1p. The amount of mt-Hsp70/luciferase complex rapidly decreased in mitochondria from the *Δmdj1* strain after shift to 40°C (Fig. 2B). In contrast, a transient increase of the complex was observed in mitochondria from the isogenic rho°, as in wild-type mitochondria (compare with Fig. 2A).

Taken together, the results demonstrate that at elevated temperature mt-Hsp70 transiently associates with a thermolabile substrate protein in the mitochondrial matrix. This transient association appears to strictly require both functional Mdj1p and Mge1p.

3.2. *Aggregation of firefly luciferase in mitochondria with mutant mt-Hsp70 and Mge1p*

Luciferase aggregation was monitored in mitochondria containing conditional mutant forms of either mt-Hsp70 (Ssc1p) or Mge1p, and in mitochondria lacking Mdj1p. Ssc1-2p carries a mutation in the substrate binding domain leading to prolonged association with substrate proteins [20]. Ssc1-3p, harbors a mutation which affects the ATPase activity which affects the capacity of the mutant protein to interact with substrate protein [20,23]. Luciferase was imported into wild-type and mutant mitochondria at 25°C. After import, mitochondria were shifted to 40°C, a temperature which leads to the induction of the mutant phenotype in *ssc1-2* and *ssc1-3* mitochondria and aggregation of luciferase (see Fig. 1). No significant difference of the aggregation kinetics was observed when wild-type and mutant mitochondria were compared (Fig. 3A). This observation was unexpected since altered kinetics of luciferase aggregation were anticipated in *ssc1-3* and *ssc1-2* mitochondria as both mutations had been demonstrated before to lead to inreased association of unfolded proteins with the mt-Hsp70 [20,23]. Taken together the results suggest that either mt-Hsp70 is either dispensible for the transient prevention of heat-induced protein aggregation or that the specific mutant alleles studied

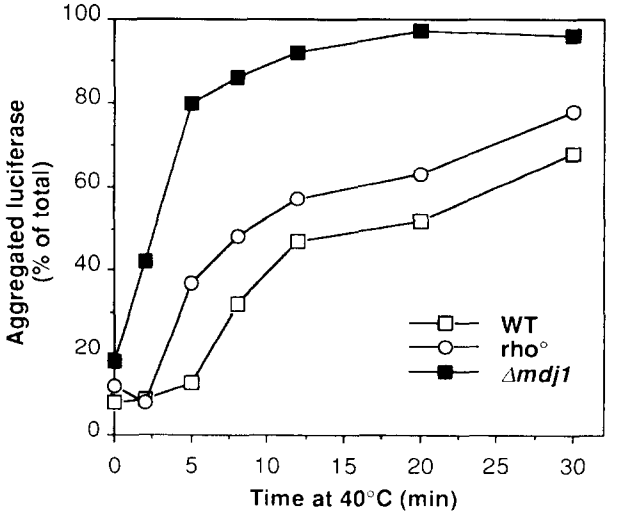

Fig. 4. Kinetics of luciferase aggregation are enhanced in mitochondria from the *Δmdj1* strain. Aggregation kinetics of luciferase was followed in mitochondria from the *Δmdj1* strain, the isogenic rho° and the wild-type strains as described above for Fig. 3A.

here do not affect the capability of mt-Hsp70 to contribute to luciferase solubility under denaturing conditions.

In order to distinguish between these two possibilities, luciferase aggregation was investigated in mitochondria with temperature-sensitive mutant forms of Mge1p, Mge1-2p and Mge1-3p. Mutations in Mge1p have been demonstrated to reduce the capability of mt-Hsp70 to interact with substrate proteins during import [17]. In case mt-Hsp70 is needed for protection against thermal denaturation by associating with denaturing proteins this process should rely on the presence of functional Mge1p. With both, mutant and wild-type mitochondria no difference in luciferase solibility was detected (Fig. 3B). These data suggest that functional mt-Hsp70 is not essential for transient protection against heat-induced protein aggregation.

3.3. *Role of Mdj1p in preventing heat-induced aggregation of luciferase*

Aggregation of luciferase was monitored in mitochondria from the *Δmdj1* strain. For control, aggregation kinetics were analyzed also in mitochondria from the isogenic wild-type strain and the isogenic rho° strain. Luciferase underwent a much more rapid aggregation in *Δmdj1* mitochondria as compared to the control mitochondria (Fig. 4). From these observations it is evident that Mdj1p plays a central role in the maintenance of the soluble state during heat stress.

4. Discussion

After shift to elevated temperature, transient increase of the complex mt-hsp70/luciferase was observed. The association of luciferase with mt-Hsp70 required the presence of both functional Mge1p and Mdj1p. These findings can be explained in the light of previous studies on the function of bacterial DnaK, DnaJ and GrpE proteins: elevated temperature induces partial unfolding of the thermolabile protein which thereby becomes a substrate for mt-Hsp70. Mge1p acts very likely in an analogous manner to its prokaryotic homologue, GrpE, and mediates exchange of nucleotides bound to mt-Hsp70. Consequently one function of Mge1p would be to recycle mt-Hsp70 to the ATP-bound form which is competent to bind unfolded substrate [17]. Lack of functional Mge1p then leads to insufficient recycling of mt-Hsp70. We have recently shown that mutations in Mge1p strongly reduce the binding of mt-Hsp70 to translocating polypeptide chains on their passage into the mitochondrial matrix [17]. Similar to its role in import, at elevated temperature, Mge1p apparently regenerates the ATP-form of mt-Hsp70 which is competent to bind to unfolded substrate proteins.

In addition to Mge1p, Mdj1p is needed for the transient increase of the mt-Hsp70/luciferase complex under denaturing conditions. Mdj1p appears to facilitate binding of mt-Hsp70 to substrate proteins. Mdj1p may do so by exerting a targeting function for mt-Hsp70 and by promoting hydrolysis of ATP bound to mt-Hsp70 that is associated with substrate proteins, similar to what has been proposed for the bacterial homologue [8,9]. Upon ATP hydrolysis, mt-Hsp70 would undergo a transition to the high affinity state to bound substrate. In this way, Mdj1p may contribute to the formation of a more stable complex of mt-Hsp70 with unfolded substrate protein.

In view of such a behaviour of mt-Hsp70 in the binding to unfolded substrate it was unexpected that the loss of solubility was not accelerated in mitochondria harboring defective forms of mt-Hsp70 or Mge1p. These observations suggest that neither functional mt-Hsp70 nor Mge1p are essential for transient prevention of heat-induced aggregation of luciferase. This conclusion is in agreement with the observation that the kinetics of luciferase aggregation were not affected by the intramitochondrial ATP level (unpublished observations).

In contrast, Mdj1p plays a central role in the transient prevention of heat-induced aggregation of luciferase. It appears that Mdj1p without the aid of mt-Hsp70 and Mge1p can prevent aggregation. Interestingly, DnaJ from *E. coli* was reported to be able to maintain the soluble state of luciferase in vitro [5].

Taken together, our data provide insight into the mechanisms by which mt-Hsp70 and its cohort chaperones stabilize matrix-located proteins against heat-induced aggregation: with the help of its cohort chaperones, mt-Hsp70 binds to unfolded luciferase and either confers transient stabilization or initiates refolding. Yet binding to mt-Hsp70 is not mandatory for transient maintenance of solubility at least in case of luciferase. The major player in this case appears to be Mdj1p. mt-Hsp70 cannot substitute for Mdj1p in this function. This is to our knowledge the first in vivo evidence that a DnaJ protein can act as a chaperone by itself. The independence of Mdj1p from mt-Hsp70/Mge1p in its protective function against aggregation may be due to a certain substrate specificity displayed by Mdj1p. Other substrate proteins may require other components besides Mdj1p for being protected against aggregation. It will be interesting to find out which determinants of a given substrate protein are responsible for the interaction with chaperones.

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