Purification and Biochemical Properties of Saccharomyces cerevisiae’s Mge1p, the Mitochondrial Cochaperone of Ssc1p*

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Previous biochemical and genetic studies have demonstrated the universal conservation of the DnaK (Hsp70) chaperone machine. Its three members, DnaK, DnaJ, and GrpE, in Escherichia coli work synergistically to promote protein protection, disaggregation, and import into the various organelles. In the mitochondria of Saccharomyces cerevisiae the three corresponding members are designated as Ssc1p, Mdj1p, and Mge1p, respectively. The MGE1 gene was previously cloned by us and others, and its product has been shown to be absolutely essential for protein transport into mitochondria and hence cell viability. To better understand its biological role, we have proceeded to overexpress and purify the mature Mge1p in E. coli through the construction of the appropriate vector clone. Mge1p has been shown to functionally substitute for its E. coli GrpE counterpart in a variety of its biological functions, including suppression of the bacterial temperature-sensitive phenotype of the grpE280 mutation, formation of a stable complex with DnaK, stimulation of DnaK’s ATPase activity, and the refolding of denatured luciferase by the DnaK/DnaJ cochaperone proteins. Thus, the function of the GrpE homologues appears to be highly conserved across the biological kingdoms.

Over the last few years, it has become apparent that Escherichia coli has evolved a sophisticated way of “sensing” the intracellular presence of excess “unfolded” proteins and dealing with this problem by appropriately raising the intracellular levels of molecular chaperone proteins (1). Molecular chaperone proteins are capable of binding to various non-native forms of proteins, thus preventing them from aggregation and favoring their proper folding (reviewed in Refs. 2–4). Many of the molecular chaperone members work together, in a synergistic way, to carry out their various biological functions in a more effective way, resulting in their designation as molecular chaperone “machines” (5).

The E. coli DnaK chaperone machine is such an example and is composed of three universally conserved members, DnaK, DnaJ, and GrpE (5). The DnaK chaperone machine not only protects unfolded polypeptides from aggregation, but is also capable of disaggregating certain heat-inducible protein aggregates (reviewed in Ref. 1). Two of its members, DnaK and DnaJ, are bona fide chaperone proteins that bind separately or synergistically to their various protein substrates. In addition to its role as a chaperone, DnaJ can catalytically accelerate the hydrolysis of DnaK-bound ATP, whereas GrpE causes the release of all DnaK-bound nucleotides (6–8). Although the exact mechanism by which GrpE causes the release of DnaK-bound nucleotide is not known, a direct, physical interaction between the two proteins has been inferred by a variety of means, including (a) the demonstration by affinity chromatography of a physical complex that is resistant to 2 M KCl (9), (b) immunoprecipitation (10), (c) glutaraldehyde cross-linking (11, 12), and (d) genetic means, as judged by allele-specific suppression studies (10, 13).

Previous work had shown that Saccharomyces cerevisiae mitochondrial proteins possess both a DnaK counterpart, termed Ssc1p, and a DnaJ counterpart, termed Mdj1p (14). The Ssc1p protein had been shown to be absolutely important for the transport of proteins into mitochondria (15), whereas Mdj1p was shown to be involved only in mitochondrial protein folding and protection against heat denaturation and aggregation (14).

Recently, three laboratories have independently reported the cloning of the S. cerevisiae gene that codes for the GrpE homologue and/or the purification of its product (16–18). As a consequence, the gene has appeared under various names in the literature, namely GRPE (16), YGE1 (17), and MGE1 (18). In this work we will refer to the gene as MGE1 and to its protein product as Mge1p. The GrpE mitochondrial homologues of S. cerevisiae and mammals were also purified based on their ability to form a tight complex with their respective Hsp70 mitochondrial protein, the DnaK analogue of E. coli (16, 19, 20). The deduced amino acid sequence of Mge1p shows approximately 30% identity to its bacterial counterparts, retaining all five distinct and highly conserved motifs, characteristic of the GrpE family (21, 22). The S. cerevisiae Mge1p protein has been shown to assist Sac1p, the resident mitochondrial Hsp70 homologue, both for the translocation and subsequent folding of various mitochondrially imported proteins (18, 20, 23, 24). The importance of the Mge1p protein in S. cerevisiae growth is exemplified by the fact that its encoding gene cannot be deleted under all growth conditions tested (16, 18).

Here, we report the purification of Mge1p, following the appropriate cloning of its encoding gene, and its overexpression in an E. coli strain that lacks the endogenous GrpE protein homologue and, in addition, lacks the wild type DnaK protein. The properties of the purified Mge1p have been extensively analyzed vis à vis its interaction with DnaK and its ability to assist the rejuvenation of denatured luciferase.

MATERIALS AND METHODS

Bacterial Strains—The E. coli DA16 (B178 grpe280 phe::Tn10 (25)) and DA262 (C600 dnaK103 thr::Tn10 grpe::Cam (25)) bacterial strains were used for genetic complementation studies and for protein purification, respectively.

Cloning of the MGE1 Gene and Construction of NH2-terminal-encoding Variants—The E. coli grpe gene was cloned under the inducible arabinose promoter of the pBAD vector (26). To do this, the genomic

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The yeast MGE1 gene was cloned from a previous pKSMGE1 plasmid (16) into the EcoRI and XhoI sites of the same pBAD vector, thus giving rise to plasmid OD23. Starting with plasmid OD23, two deleted variant plasmids, OD24 and OD25, were constructed. Plasmid OD24 lacks the first 72 base pairs of the MGE1 encoding sequence, whereas plasmid OD25 lacks the first 129 base pairs of the MGE1 encoding sequence. OD24 and OD25 were cloned into the same expression vector, following the way (see Fig. 1A). Using the U-DNA mutagenesis kit from Boehringer Mannheim, a DNA segment encoding for the EcoRI-NdeI restriction sites was introduced, either at the 72-bp position or the 129-bp position of the MGE1 gene carried on plasmid OD23. For this purpose, the 5'-Gcataagggatcataaggtccttgg-3' and 5'-cataagaaggtgccaatgta-3' DNA primers were constructed and used. Following this, the plasmids were digested with EcoRI and ligated, and the DNA junction regions of plasmids OD24 and OD25 were verified by DNA sequencing, using the Sanger et al. dideoxy sequencing method (13). Plasmids OD23, OD24, and OD25 were transformed into E. coli strain DA262 (25), which is deleted for the grpE gene and encodes for the truncated DnaK103 protein, to ensure the lack of contamination by the GrpE or DnaK endogenous proteins of E. coli. Transformants were grown overnight in LB Bertani (10 g of NZM, 5 g of NaCl, and 1.5 ml of yeast extract, 5 g of NaCl, and 0.3 ml of 10 x NaOH/liter) to an A600 of ~1.0. The synthesis of plasmid-encoded proteins was preferentially induced by adding arabinose [0.5% (w/v) final concentration] and the bacterial cultures grown for an additional 2 h with aeration at 37 °C. An aliquot of each culture was processed by SDS-PAGE (15% (w/v) polyacrylamide), immunoblot experiments were carried out using Mgp1p-specific rabbit antisera (1,300 dilution; kindly provided by Prof. G. Schatz, Biozentrum, Basel, Switzerland), and was developed with aldehyde-phosphatase-conjugated anti-rabbit IgG as a secondary antibody (Bio-Rad Immun-Blot assay kit).

**Purification of Mgp1p-43**—Plasmid OD25, encoding Mgp1p-43, was transformed into DA262 bacteria, thus giving rise to bacterial strain OD133. Approximately 10 ml of an overnight culture of OD133 bacteria, growing in LB broth supplemented with 100 µg/ml of ampicillin, was used to inoculate five flasks containing 1 liter of the same medium. The cultures were grown with aeration at 37 °C to an A600 of ~1.0, at which time arabinose was added to a final concentration of 0.5%. The cultures were then grown for an additional 2 h, harvested, rinsed with sucrose buffer (50 mM Tris, pH 8.0, 10% (w/v) sucrose), resuspended into 20 ml of the same buffer, and stored at ~80 °C.

The frozen cells were thawed slowly at 4 °C, 1 ml of lysis buffer (0.92 g of spermidine, 1 ml of 1 M DTDT, 2 ml of 0.5 M EDTA, and 3.26 g of ammonium sulfate, 20 ml final volume) was added slowly, and the volume was brought to 100 ml with sucrose buffer. Following this, 4 ml of a freshly prepared lysozyme solution (10 mg of lysozyme/ml of sucrose buffer) was added slowly, and the mixture was left on ice for 45 min. The mixture was then incubated at 37 °C for 5 min, and the cell debris was removed by centrifugation in a fixed angle 35 Beckman ultracentrifuge rotor at 30,000 rpm for 30 min at 4 °C. Following this, 35 g of solid ammonium sulfate were added per 100 ml of supernatant liquid over a period of 15 min, under constant stirring and incubated at 4 °C for 30 min. Precipitated proteins were harvested following centrifugation (20 min at 20,000 rpm, at 4 °C, using a fixed angle 35 Beckman ultracentrifuge rotor), and the pellet was resuspended in 60 ml of buffer A (50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 10% (w/v) sucrose, 10 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride) and dialyzed against the same buffer overnight at 4 °C. The dialyzed proteins were applied onto a Q-Sepharose column (2.5 × 7 cm), previously equilibrated in buffer A. The column was washed with 3 column volumes of buffer A, and the proteins were eluted with a linear gradient of 0.05–0.4 M KCl. Following the separation of proteins by SDS-PAGE (15% (w/v) acrylamide), the presence of the Mgp1p-43 protein in individual fractions was detected by immunoblotting. The fractions that were enriched in Mgp1p-43 protein were pooled and dialyzed overnight against buffer A at 4 °C. The dialyzed was loaded onto a blue Sepharose Fast Flow column (1.5 × 5 cm), equilibrated with buffer A, and eluted with a 100-ml gradient of 0.05–0.4 M NaCl. Those fractions that contained the most highly purified Mgp1p-43 protein were pooled and dialyzed overnight against buffer B (10 mM imidazole, pH 6.9, 10 mM MgCl2, 1 µM EDTA, 10% (w/v) sucrose, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol) at 4 °C. Fractions containing highly purified Mgp1p-43 protein, as judged on SDS-PAGE, were dialyzed overnight against buffer C supplemented with 1 µM NaCl at 4 °C. The dialyzed proteins were loaded onto a phenyl-Sepharose column (1.5 × 12 cm), previously equilibrated with buffer A, containing 1 µM NaCl. The column was washed with 3 volumes of the same buffer and eluted with a linear gradient of 1.0–0.0 M NaCl in buffer A.

The yeast GrpE wild-type protein was purified under the same conditions described above for Mgp1p-43 until the blue Sepharose column step, using extracts prepared from strain DA262, transformed with plasmid OD1. Those fractions that contained highly purified GrpE protein were pooled together and dialyzed overnight against buffer D (100 mM Tris/HCl, pH 7.6) at 4 °C. The dialyzed fractions were loaded onto a Superdex-200 gel filtration column (Pharmacia K16/60), previously equilibrated with buffer D, and the proteins were concentrated using a Centricon 10 concentrator. The buffer was exchanged by using 3 × 15 ml of buffer C (40 mM Hepes pH 7.6, 1 mM EDTA, 10% (w/v) sucrose, and 50 mM KCl), and the purified proteins were stored at −80 °C. The protein concentrations were estimated using the Bradford reagent assay (2).

**Analytical Gel Filtration Chromatography**—Aliquots of highly purified proteins (200 µl; 2.5 µg of total protein) were loaded at 4 °C onto a 96-well microtiter plate in 50 µl of PBS buffer and incubated at room temperature for 2 h. The wells were then washed twice with 200 µl of PBS. The unreacted groups were blocked at room temperature with 200 µl of 5% powdered milk in PBS for 2 h, followed by two washes with 200 µl of PBS. The wells were rinsed once with 200 µl of buffer A (25 mM Heps, pH 7.6, 150 mM KCl, 5 mM MgCl2, 1 mM EDTA, 0.1 mM DTT, 0.1 M Na3C4H4O6, 25 g of spermidine, 10 mM (imidazole, pH 6.9, 10 mM MgCl2, 1 mM EDTA, 10% (w/v) sucrose, and 50 mM KCl), and the purified proteins were stored at −80 °C. The protein concentrations were estimated using the Bradford reagent assay (2).

**Glutathaldehyde Cross-linking**—Cross-linking of proteins with glutathaldehyde was carried out essentially as described previously (12, 27).

**Enzyme-linked Immunosorbent Assay (ELISA)**—The procedure employed was a modified version of that used by Marszalek et al. (28) and Wu et al. (12). Briefly, either GrpE or Mgp1p-43 protein was added to a 96-well microtiter plate in 50 µl of PBS buffer and incubated at room temperature for 2 h. The wells were then washed twice with 200 µl of PBS. The unreacted groups were blocked at room temperature with 200 µl of 5% powdered milk in PBS for 2 h, followed by two washes with 200 µl of PBS. The wells were rinsed once with 200 µl of buffer A (25 mM Heps, pH 7.6, 150 mM KCl, 5 mM MgCl2, 1 mM EDTA, 0.1 mM DTT, 0.1 M Na3C4H4O6, 25 g of spermidine, 10 mM (imidazole, pH 6.9, 10 mM MgCl2, 1 mM EDTA, 10% (w/v) sucrose, and 50 mM KCl), and the purified proteins were stored at −80 °C. The protein concentrations were estimated using the Bradford reagent assay (2).

**ATPase Activity**—The ATPase activity of DnaK was measured by the conversion of [γ-32P]ATP (0.1 µCi with a specific activity of 5,000 Ci/mmol; Amersham Corp.) to γ-32P-ATP, at 30 or 4°C, essentially as described previously (6). The reaction was prepared on ice and initiated by the addition of the ATP. The mixture was heated to the indicated temperature. At various times, 0.5-µl aliquots were removed and spotted onto polyethyleneimine-cellulose thin layer sheets. The ATP and P, forms were separated by one-dimensional chromatography using a solution of 1 M formic acid/l M LiCl (1:1%). The ATP and P, positions were identified by using an excess of a cold ATP, mixture during the chromatographic step, cut out, and the amount of radioactivity in each spot was determined by liquid scintillation counting. The initial velocity of the ATPase reaction was calculated using the linear regression method described by Liberak et al. (6).

**Nucleotide Release Assay**—The DnaK [γ-32P]ATP or ADP complex was quickly isolated on a Bio-Rad P60 column (0.2 × 3 cm), as described
by Liberek et al. (8), and frozen in liquid nitrogen. The isolated complex had a final concentration of 0.52 μg of DnaK/ml and a specific activity of 8,880 cpm/μg of DnaK. The DnaK α-32P nucleotide complex prepared in this manner was incubated with either GrpE or Mge1pΔ43 protein in buffer A (50 mM Tris/HCl, pH 7.8, 50 mM NaCl, 50 mM KCl, 10 mM MgCl2, 2 mM DTT) for 1 min at 30 °C and then quickly loaded onto a P80 column (0.2 × 3 cm), previously equilibrated with buffer A, in order to separate the unbound nucleotides from those bound onto DnaK. Two drop fractions were collected, and the radioactivity content of 4 μl of each fraction was determined by liquid scintillation counting.

**Firefly Luciferase Refolding Assay**—The renaturation of denatured firefly luciferase was carried out essentially as described by Szabo et al. (7). Briefly, firefly luciferase at 25 μM was denatured in a buffer containing 30 mM Tris/HCl, pH 7.4, 6 mM guanidinium HCl, and 5 mM DTT for 30 min at 22 °C. The denatured luciferase was diluted into a 100 μl mixture reaction containing 10 mM MOPS, pH 7.2, 50 mM KCl, 5 mM MgCl2, 1 mM ATP, 0.5 μM DnA1, and 1.25 μM DnaK. Following a 10-min incubation at 25 °C, the refolding reaction was initiated by the addition of either GrpE or Mge1pΔ43 protein to a final concentration of 1.25 μM. The resulting luciferase activity was measured using the Promega luciferase assay, followed by liquid scintillation counting.

**RESULTS**

**Expression and Purification of the Mge1pΔ43 Protein, the S. cerevisiae GrpE Homologue**—Previously, we identified and cloned the *S. cerevisiae* MGE1 gene, encoding for the mitochondrially located protein homologue of *E. coli*’s GrpE. Mge1p was shown to intimately interact with Ssc1p, the mitochondrially located DnaK protein homologue (16, 20, 23). Although Mge1p is cleaved during its import into mitochondria, the exact cleavage site of its mitochondrial signal sequence has not been definitively identified yet, due to the fact that its amino-terminal residue is blocked (16). This, coupled with the fact that mitochondrial leader sequences are very loosely defined, has led to the ambiguity as to the exact cleavage site of Mge1p. Based on the alignment of Mge1p to that of GrpE, two amino acid residues, located at position 24 and 43, respectively, have been proposed as potential cleavage sites for the mitochondrial matrix endopeptidase (16, 18).

Because of this ambiguity, we proceeded to construct two variants of *MGE1* (starting with plasmid OD23), which encode for proteins beginning at amino acid residues 24 (plasmid OD24) or 43 (plasmid OD25), respectively. This was done by inserting the EcoRI/NdeI restriction sites into nucleotide positions +77 and +129 of the *MGE1* encoding sequence (see Fig. 1A and “Materials and Methods”) and eliminating the DNA between the two EcoRI sites, by first digesting with the EcoRI restriction enzyme and religating (Fig. 1A).

The three plasmid constructs (OD23, OD24, OD25), along with the parental plasmid vector, were introduced separately into the DA16 strain (*E. coli grpE280* mutant bacteria) and tested for their ability to complement the GrpE280 temperature-sensitive phenotype at 43 °C (Ang et al., 1986). It was found that the OD24 and OD25 constructs allowed grpE280 mutant bacteria to grow at 43 °C, whereas the OD23 construct and the parental plasmid vector did not (result not shown). In order to determine whether the three Mge1p variant proteins were adequately expressed in *E. coli*, we introduced the three plasmids into *E. coli* strain DA259, which is deleted for the chromosomally encoded *grpE* gene (25), and induced the synthesis of the yeast *Mge1p* protein with the addition of 0.5% arabinose. Proteins were separated by SDS-PAGE and a Western immunoblot was carried out using anti-Mge1p antibodies, as described under “Materials and Methods.” Fig. 1B shows that no protein antigenically related to Mge1p was detected in bacteria carrying either the plasmid vector or the OD23 plasmid. On the contrary, bacteria carrying the OD24 plasmid expressed two immunologically related proteins upon arabinose induction, one with an apparent mobility of a protein of approximately 30,000 Da and the other of 28,000 Da. Pulse-chase experiments, using [35S]methionine showed that the 28,000- and 30,000-Da protein are synthesized de novo (results not shown).

![FIG. 1. Construction and expression of various MGE1 alleles in E. coli.](http://www.jbc.org/)

**A** A, three *MGE1* alleles were cloned into the *EcoRI* and *XhoI* sites of the pBAD vector, so that their expression is under the tightly regulated, inducible arabinose promoter. 1) Plasmid OD23 (the entire *MGE1* gene coding sequence), 2) plasmid OD24, and 3) plasmid OD25. The first 24-amino acid and 43-amino acid coding sequences were removed in plasmids OD24 and OD25, respectively, by the insertion of the *EcoRI*-NdeI sites into the *MGE1* gene (see “Materials and Methods” for details). The encoded amino acids (single letter) in the vicinity of the putative cleavage sites are indicated. B, expression of the various *MGE1* alleles were analyzed by Western immunoblot technology. The proteins were separated by means of 15% (w/v) SDS-PAGE, then electrotransferred and immunodetected using specific anti-Mge1p antibodies (see “Materials and Methods” for details). Lane 1, pBAD vector; lane 2, OD23; lane 3, OD24; lane 4, OD25.
Formation of a Stable Complex between Mge1p43 and DnaK—A loop consisting of 6 amino acid residues in the ATPase domain of DnaK (residues 29–34 in E. coli’s DnaK) has been shown to be essential for its stable binding to GrpE (31). Since this loop is extremely well conserved among prokaryotic and mitochondrial GrpE family members (with only a single amino acid change between DnaK and Ssc1p, the mitochondrial Hsp70 homologue), we tested the ability of Mge1p43 to interact with DnaK in vitro.

The first method attempted was cross-linking by glutaraldehyde. Using this method, Ang (11) and Osipiuk et al. (27) had previously shown that a dimer of GrpE interacts with a monomer of DnaK. As can be seen in Fig. 3, lanes 3 and 4, glutaraldehyde cross-linking showed that either GrpE or Mge1p43 alone behaved predominately as dimers in solution, although some higher oligomeric species could also be detected. As expected, glutaraldehyde non-treated GrpE protein behaved as monomer under identical SDS-PAGE conditions (result not shown). In the presence of DnaK, the major cross-linked product had an apparent molecular mass greater than 97,400 Da, which corresponds to two monomers of Mge1p43 and one monomer of DnaK (Fig. 3, lane 7). Judging by the proportion of free, non-cross-linked DnaK, it appears that, under these conditions, Mge1p43 interacts with DnaK with the same efficiency as GrpE does. The assignment and identity of the proteins in the various cross-linked complexes was verified by immunoblot analysis (results not shown). As a control for the specificity of binding, a truncated E. coli GrpE protein, GrpE41, was used, which lacks the 20 carboxyl-terminal amino acid residues, and, as a consequence, interacts very weakly with DnaK (Fig. 3, lanes 2 and 8).

In order to further characterize the interaction between Mge1p43 and DnaK, the effect of high salt and various nucleotides on complex formation was investigated using the ELISA technique (see “Materials and Methods” for details). Although the Mge1p43-DnaK complex formation was observed in the presence of 1 M KCl (Fig. 4B), no such complex was observed in the presence of either 2 mM ATP or ATPγS, its non-hydrolyzable analogue. In all these respects, the Mge1p43 and GrpE proteins behaved identically (Fig. 4A).

Mge1p43 Stimulates DnaK’s ATPase Activity—It was
shown previously that DnaK possesses a very weak ATPase activity, approximately one ATP molecule hydrolyzed every 5 min per DnaK monomer (6, 32). This weak ATPase activity can be accelerated at least 50-fold in the presence of the DnaJ and GrpE “cohort” proteins (6). To further delineate the extent of conservation of the molecular activities of Mge1p43 and GrpE, the ability of the former to increase the rate of DnaK’s ATPase activity was tested. Fig. 5 shows that at 30°C Mge1p43 was identical to GrpE in its ability to stimulate DnaK’s ATPase activity. In contrast, at 43°C, GrpE, but not Mge1p43, was capable of stimulating DnaK’s ATPase activity (Fig. 5).

The precise function of GrpE in stimulating DnaK’s ATPase activity has been traced to its ability to release the DnaK-bound nucleotide, thus facilitating DnaK’s recycling by acting as nucleotide exchanger for DnaK (6). In order to determine whether Mge1p43 can also accomplish this function, it was incubated with a DnaK-[γ-32P]ATP preparation (actually during the time it takes to prepare the DnaK-ATP complex, most of the ATP is hydrolyzed to ADP, still bound to DnaK) (6). Fig. 6 shows that Mge1p43, similarly to GrpE, releases all DnaK-bound nucleotides, confirming that Mge1p43 can also act as a nucleotide exchange factor for DnaK.

Refrolding of Denatured Luciferase by DnaK, DnaJ, Mge1p43—Previous studies have led to the conclusion that the principal biological role of the GrpE protein is to destabilize the DnaK-substrate-DnaJ complex, by promoting the ADP/ATP exchange cycle of the DnaK chaperone (4). Without such assistance from GrpE, the DnaK chaperone machine operates at a very low efficiency. In order to substantiate the functioning of Mge1p43 in the DnaK reaction cycle, we analyzed its ability to assist the DnaK/DnaJ chaperones in the refolding of denatured firefly luciferase (7). To do this, luciferase was first denatured in 6 M guanidinium HCl and diluted 100-fold in the presence of the DnaK/DnaJ chaperones and MgATP. The degree of correct refolding was determined by assaying for luciferase activity, in the presence or absence of GrpE or Mge1p43. As seen in Fig. 7, the addition of either GrpE or Mge1p43 greatly stimulated the correct refolding of guanidinium-denatured luciferase, indicating that either protein can function with the DnaK/DnaJ chaperones to promote correct protein folding. Interestingly, when either GrpE or Mge1p43 were added at the same time as DnaK and DnaJ, the correct refolding of inactivated luciferase was greatly reduced (less than 20% activity recovered; result not shown). This last result suggests that the efficient action of the GrpE or Mge1p43 protein requires the prior formation of a stable DnaK-luciferase-DnaJ complex and is consistent with the proposed role of GrpE in destabilizing the DnaK-substrate-DnaJ complex (7, 33).

DISCUSSION

Both the structure and function of the DnaK (Hsp70) chaperone machine have been universally conserved (1, 3). In prokaryotes, the DnaK chaperone machine consists of three members, DnaK, DnaJ, and GrpE. Two of its members, DnaK and DnaJ, are bona fide molecular chaperones, capable of binding to certain unfolded or aggregated proteins either separately or
synergistically, thus protecting them from aggregation or helping them to maintain a properly unfolded state, required for protein export. The DnaK/DnaJ interactions are most likely very elaborate. The first contact between the two chaperones is catalyzed by the so-called “J-domain,” a highly conserved, approximately 70-amino acid-long sequence, constituting the signature of the DnaJ proteins (34, 35). An intact J domain is absolutely essential for stimulating the hydrolysis of the DnaK-bound ATP (36).

In contrast to DnaK and DnaJ, the GrpE protein does not possess any known chaperone activities. Its only known biological role is that of assisting DnaK in carrying out its chaperone functions. This conclusion is derived from the following lines of evidence (a) the grpE gene cannot be deleted in *E. coli* under various experimental conditions, yet it can be deleted in all genetic backgrounds that have been previously selected for bacterial growth in the absence of DnaK function (25); (b) the GrpE protein binds tightly to DnaK and this association is readily disrupted in the presence of ATP (9); (c) the GrpE protein acts like a nucleotide exchange factor for DnaK, inasmuch as it causes the release of either ADP or ATP bound to DnaK (6); and (d) the presence of GrpE protein interferes with the stable association of polypeptide substrates to DnaK, even in the absence of ATP (27). This last property does not appear to be due to a “steric hindrance” effect by GrpE, since the transient presence of a DnaK-substrate-GrpE complex can be readily demonstrated (27). Very likely then, GrpE accomplishes its biological function in two distinct ways. First, by acting like a nucleotide exchanger for DnaK, it promotes the exchange of ADP for ATP, which in turn can destabilize the DnaK-substrate complex (37, 38). Second, when bound to DnaK it may affect DnaK’s tertiary structure, thus disfavoring a stable DnaK-substrate association.

The *S. cerevisiae* *MGE1* gene, encoding for the mitochondrially located Mge1p, the GrpE homologue, has been recently cloned independently by three different laboratories (16–18). Like its *grpE* counterpart of *E. coli*, the *MGE1* gene is absolutely essential for cell viability. Mutational analysis has demonstrated that functional Mge1p is required for polypeptide import into mitochondria (24). Most likely, Mge1p assists in polypeptide import by interacting with Sac1p, the mitochondrially located DnaK homologue of Yeast reviewed by Schatz and Dobberstein (15). By analogy with the *E. coli* system, the function of Mge1p has been assumed to be a nucleotide exchange factor for Sac1p. This assumption was experimentally verified by us in the work reported here (see below). Interestingly, Mdj1p, the mitochondrially located DnaJ homologue (14) does not participate directly in polypeptide import into mitochondria. Instead, its role is assumed by the membrane-bound Tim44 (Mim44 or Isp45) protein (reviewed in Ref. 15).

In this work we have begun an investigation on the biochemical properties of Mge1p. In order to obtain enough biological material for biochemical studies, we proceeded to overexpress Mge1p in *E. coli*. Since all mitochondrially imported polypeptides possess an amino-terminal leader sequence that is cleaved, we designed a vector that overproduces Mge1pΔ43, deleted for the first 43 amino-terminal residues encoded by *MGE1*. Mge1pΔ43 has the same mobility as mitochondrially located Mge1p, suggesting that the proposed import cleavage site of Lalaraya et al. (18) is the one used in *vivo* and not the one previously proposed by us (16). Interestingly, the full-length Mge1p, containing an intact presequence, when expressed in *E. coli* did not accumulate to an appreciable extent, suggesting that it is rapidly degraded. In contrast, Mge1pΔ43 was fully stable, thus accumulating to high intracellular levels.

The Mge1pΔ43 protein was shown to behave, both qualitatively and quantitatively, in a fashion identical to that of GrpE of *E. coli*, at least four points (a–d) namely (a) its native size, as judged by its elution profile on a size column; (b) its ability to stimulate DnaK's ATPase activity by acting as a nucleotide exchange factor. An interesting finding made here was that, in contrast to GrpE, Mge1pΔ43 stimulated efficiently DnaK's ATPase at 30°C, but not at 43°C. This result may not be surprising, since *S. cerevisiae* is not capable of growth at 43°C, but it is surprising when considering that Mge1pΔ43 complemented the temperature-sensitive phenotype of *grpE*Δ280 mutants at 43°C. This result, coupled with the observation that Mge1pΔ43 did not complement the temperature-sensitive phenotype of a *grpE* deletion, suggests that Mge1pΔ43 can form functional heterodimers with the GrpE protein of *E. coli* and that such heterodimers are biologically active, (c) its binding to DnaK as judged by glutaraldehyde cross-linking. Like GrpE, the glutaraldehyde cross-linking study showed that Mge1pΔ43 binds to DnaK predominantly as a dimer, in agreement with previous studies showing that GrpE also binds to DnaK as a dimer (11, 27), and finally (d) its participation in the proper renaturation of guanidinium-inactivated luciferase. In this biochemical assay, Mge1pΔ43 was capable of assisting the DnaK/DnaJ chaperones to the same extent as the GrpE protein of *E. coli* did. Taken together, all these results demonstrate that mitochondrial Mge1pΔ43 interacts with DnaK in a manner exactly analogous to that of GrpE and serve to highlight the evolutionary conservation of the DnaK chaperone machine.

The fact that no GrpE homologue has been detected thus far in either the cytosol or the endoplasmic reticulum of eukaryotic cells presents the question of why not? One explanation is that such GrpE homologues do exist, but have gone undetected up to now, because their complex with their corresponding Hsp70 member is not strong enough to be detected by affinity chromatography or co-immunoprecipitation. Buchberger et al. (31) have shown that the DnaK amino acid residues 28–33 are
absolutely required for stable binding to GrpE. In this respect it is interesting that amino acid residue 31 is glutamic acid in all prokaryotic and mitochondrial DnaK homologues, but the corresponding amino acid in all of the other eukaryotic counterparts is a highly conserved glutamine. Perhaps this amino acid residue plays a key role in the stabilization of the GrpE-DnaK complex. Another explanation is that GrpE is simply not required for the activation of cytosolic Hsp70s. Ziegelhoffer et al. (39) have provided a clue as to why Ssa1p may function in the absence of a GrpE-like cytosolic homologue. These authors showed that although Ssa1p binds tightly to its ATP substrate, it releases ADP spontaneously. As a consequence, the isolated Ssa1p-ATP complex is void of ADP, in sharp contrast with DnaK, where approximately equimolar amounts of both the ATP- and ADP-bound forms are found (6). Thus, the rate-limiting step in Ssa1p's ATPase reaction appears to be the hydrolysis of the bound ATP, and, hence, the usefulness of the putative GrpE-like nucleotide exchange factor would be limited in the Ssa1p system. Consistent with this conclusion, Levy et al. (40) and Freeman et al. (41) have shown that cytosolic Hsp70 proteins, together with their corresponding cytosolic DnaJ homologues, can reactivate denatured proteins, in a GrpE-independent reaction. In contrast, E. coli's DnaK and DnaJ proteins absolutely require the presence of GrpE to carry out this interesting biological reaction (7). Finally, another possibility is that additional regulatory factors may exist in the cytosol, which are not related in sequence to GrpE, but still modulate the biochemical properties of some of the cytosolic Hsp70s. For example, Höhfeld et al. (42) have shown that the cytosolic Hip protein interacts with the highly conserved ATPase domain of human Hsc70, thusly modulating its biochemical properties.

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