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Mge1 Functions as a Nucleotide Release Factor for Ssc1, a Mitochondrial Hsp70 of *Saccharomyces cerevisiae*

Bingjie Miao, Julie E. Davis and Elizabeth A. Craig*

Department of Biomolecular Chemistry, 1300 University Avenue, University of Wisconsin, Madison, WI 53706 USA

**Corresponding author*

Mge1, a GrpE-related protein in the mitochondrial matrix of the budding yeast *Saccharomyces cerevisiae*, is required for translocation of precursor proteins into mitochondria. The effect of Mge1 on nucleotide release from Ssc1, an Hsp70 of the mitochondrial matrix, was analyzed. The release of both ATP and ADP from Ssc1 was stimulated in the presence of Mge1, therefore we conclude that Mge1 functions as a nucleotide release factor for Ssc1. Mge1 bound stably to Ssc1 *in vitro*; this interaction was resistant to high concentrations of salt but was disrupted by the addition of ATP. ADP was much less effective in releasing Mge1 from Ssc1 whereas ATPγS and AMPPNP could not disrupt the Ssc1/Mge1 complex. Ssc1-3, a temperature sensitive *SSC1* mutant protein, did not form a detectable complex with Mge1. Consistent with the lack of a detectable interaction, Mge1 did not stimulate nucleotide release from Ssc1-3. A conserved loop structure on the surface of the ATPase domain of DnaK has been implicated in its interaction with GrpE. Since the single amino acid change in Ssc1-3 lies very close to the analogous loop in Ssc1, the role of this loop in the Ssc1:Mge1 interaction was investigated. Deletion of the loop abolished the physical and functional interaction of Ssc1 with Mge1, suggesting that the loop in Ssc1 is also important for the Ssc1:Mge1 interaction. Two mutants with single amino acid changes within the loop did not eliminate the stable binding of Mge1, yet the binding of Mge1 did not stimulate the release of nucleotides from the mutant *SSC1* proteins. We propose that the loop region of Ssc1 is important for the physical interaction between Mge1 and Ssc1, and for generation of a conformational change necessary for Mge1-induced nucleotide release.

Keywords: Hsp70; Mge1; Ssc1; nucleotide release; chaperone

Introduction

The 70-kDa heat shock proteins (Hsp70s) have been highly conserved during evolution and are present in every organism examined so far. Hsp70s are important for a variety of cellular functions, including protein folding, protein translocation across biological membranes, and protein degradation (for reviews, see Craig *et al.*, 1993; Hartl, 1996; Morimoto *et al.*, 1994). Hsp70s have two functional domains, a highly conserved N-terminal ATPase domain, which binds and hydrolyzes ATP, and a somewhat less conserved peptide binding domain (Chappell *et al.*, 1987; Wang *et al.*, 1993; Freeman *et al.*, 1995). The tertiary structure of the 44-kDa ATPase domain is similar to that of hexokinase and actin (Flaherty *et al.*, 1990, 1991; Bork *et al.*, 1992), whereas the C-terminal peptide binding domain has a unique β-sandwich structure followed by an extended structure of α-helices (Zhu *et al.*, 1996). The interaction between the two domains is critical for the function of Hsp70s (Buchberger *et al.*, 1994b, 1995). Hsp70s function as molecular chaperones by binding to short stretches of hydrophobic peptide sequences thus preventing premature folding or aggregation of partially unfolded proteins (Flynn *et al.*, 1989; Blond-Elguindi *et al.*, 1993; Gragerov *et al.*, 1994). Upon ATP binding and/or hydrolysis, bound

Abbreviations used: ATPγS, adenosine 5′-O-(3-thiotriphosphate); AMPPNP, 5′-adenylyl-β,γ-imidodiphosphate; 5-FOA, 5-fluoroorotic acid; GST, glutathione *S*-transferase; IPTG, isopropyl β-D-thiogalactopyranoside; PEI-cellulose, polyethyleneimine-cellulose; TLC, thin layer chromatography.

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peptide is released to allow for its proper folding (Palleros *et al*., 1993; McCarty *et al*., 1995; Banecki & Zylicz, 1996). This cycle of ATP binding and hydrolysis coupled to peptide binding and release is essential for the function of Hsp70s.

Several lines of evidence indicate that DnaK, an Hsp70 protein of *Escherichia coli*, functions together with two proteins, DnaJ and GrpE (reviewed by Georgopoulos *et al*., 1994). Mutations in the *dnaK, dnaJ* or *grpE* genes result in similar phenotypes; furthermore, DnaK, DnaJ and GrpE function together in a variety of *in vitro* assays, such as initiation of λ DNA replication and refolding of denatured proteins. DnaJ and GrpE exert their effects, at least in part, by modulating the ATPase activity of DnaK. DnaJ stimulates the hydrolysis of bound ATP by DnaK, whereas GrpE promotes the release of nucleotides from DnaK (Liberek *et al*., 1991a). DnaJ alone stimulates the steady-state ATPase activity of DnaK by two to tenfold, whereas GrpE alone has a minimal effect (Jordan & McMacken, 1995; McCarty *et al*., 1995). However, DnaJ and GrpE together can stimulate the steady-state ATPase activity of DnaK by up to 100-fold.

Unlike the DnaK:DnaJ interaction, GrpE binds tightly to DnaK. The complex between DnaK and GrpE is stable in the presence of high concentrations of salt, but is disrupted upon the addition of ATP (Zylicz *et al*., 1987). GrpE binds to the 44-kDa ATPase domain of DnaK, and a conserved loop structure on the surface of the ATPase domain has been implicated in the interaction of DnaK with GrpE (Buchberger *et al*., 1994a). A point mutation in this loop as well as a deletion of this loop eliminates the physical and functional interaction between DnaK and GrpE. It has been proposed that the binding of GrpE induces a conformational change in DnaK, thus triggering nucleotide release (Buchberger *et al*., 1994a), yet the mechanism of GrpE-induced nucleotide release remains to be elucidated.

Numerous eukaryotic Hsp70s and several DnaJ-related proteins have been identified in eukaryotic cells. Genetic and biochemical studies have established the functional interaction between them (reviewed by Cyr *et al*., 1994). Mge1 (also referred to as Yge1p, GrpEp), a GrpE-related protein, has recently been identified in the mitochondrial matrix of *Saccharomyces cerevisiae* (Laloraya *et al*., 1994; Bolliger *et al*., 1994; Ikeda *et al*., 1994). Mge1, which shares 34% identity with *Escherichia coli* GrpE (Laloraya *et al*., 1994), is essential for the growth of *S. cerevisiae*. Mge1 is required for normal import and maturation of nuclear-encoded mitochondrial proteins (Laloraya *et al*., 1994, 1995; Westerman *et al*., 1995).

Ssc1, an essential Hsp70 in the mitochondrial matrix, is also required for the translocation and maturation of precursor proteins, as temperature-sensitive mutations in *SSC1* result in a block of import of precursor proteins upon shifting to the non-permissive temperature (Kang *et al*., 1990; Gambill *et al*., 1993). Ssc1 has been found to be associated with precursor proteins during and after translocation (Ostermann *et al*., 1990; Scherer *et al*., 1990; Manning-Krieg *et al*., 1991), consistent with its role in the import and maturation of precursor proteins. The binding of Ssc1 to precursor proteins in transit across the mitochondrial membranes is essential for conferring the unidirectionality of the import process (Ungermann *et al*., 1994). Mge1 can be quantitatively co-immunoprecipitated with Ssc1 from isolated mitochondria, and both Ssc1 and Mge1 can be co-immunoprecipitated with a precursor protein that is trapped at the import site (Voos *et al*., 1994), suggesting a functional cooperation between Ssc1 and Mge1 in the process of protein translocation.

Because of the sequence similarity between Mge1 and GrpE, and the functional cooperation between Ssc1 and Mge1, it has been hypothesized that Mge1 functions as a nucleotide release factor for Ssc1. Here we show that Mge1 is in fact a nucleotide release factor for Ssc1. We also characterized the Ssc1:Mge1 interaction and examined the effect of mutations of the loop in Ssc1, which is analogous to the GrpE-interacting loop in DnaK, on the interaction of Ssc1 with Mge1. Based on these results, a possible mechanism for Mge1-induced nucleotide release is discussed.

Results

Mge1 is a nucleotide release factor for Ssc1

Since GrpE functions as a nucleotide release factor for DnaK, we wanted to test whether Mge1 is able to function as a nucleotide release factor for Ssc1. Consistent with the lack of an effect of GrpE on the ATPase activity of DnaK (Jordan & McMacken, 1995), Mge1 had a minimal effect on the ATPase activity of Ssc1 (data not shown). To examine more closely the interaction of Mge1 with Ssc1, an isolated step in the ATPase reaction, the ATP hydrolysis step, was analyzed by single turnover experiments. Complexes of Ssc1 and [α-^{32}P]ATP were formed at 30°C; isolation of the complex by size exclusion chromatography was carried out at 4°C to limit the hydrolysis of [α-^{32}P]ATP. The isolated Ssc1/ATP complex was then incubated at 30°C and the hydrolysis of bound ATP monitored. As shown in Figure 1A, wild-type Ssc1 hydrolyzed bound ATP with about 50% of the bound ATP being hydrolyzed within four minutes of incubation. To investigate the stability of the interaction of ATP with Ssc1, the ability of excess unlabeled ATP or ADP to quench the hydrolysis of the bound [α-^{32}P]ATP was determined. Assuming that there is no significant cooperativity in nucleotide binding to Ssc1, addition of unlabeled nucleotide should have no effect on the hydrolysis of ATP which remains bound to Ssc1. However, [α-^{32}P]ATP which is released prior to hydrolysis must then compete with the vast excess of unlabeled nucleotide for rebinding before

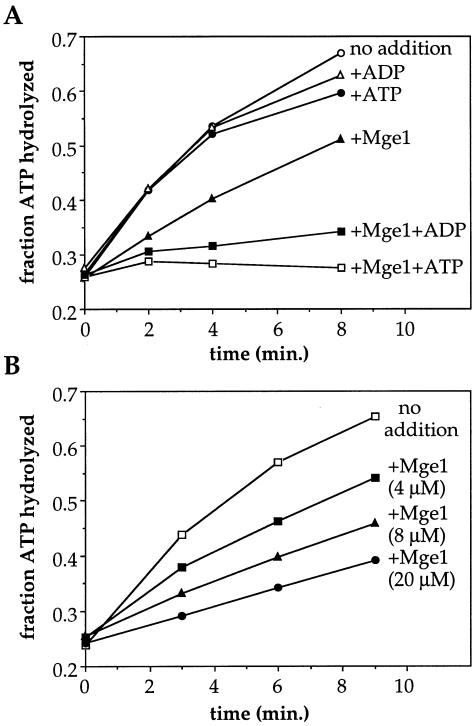

Figure 1. Effects of excess nucleotides and Mge1 on the single turnover of Ssc1/ATP complex. A, The Ssc1/ATP complex (~2 μM) was incubated at 30°C with the addition of Mge1 (4 μM), ATP (250 μM) and ADP (250 μM) as indicated. Aliquots were withdrawn at the indicated time points and the fraction of ATP converted to ADP determined as described in Materials and Methods. B, Same as in A with the indicated amount of Mge1 added.

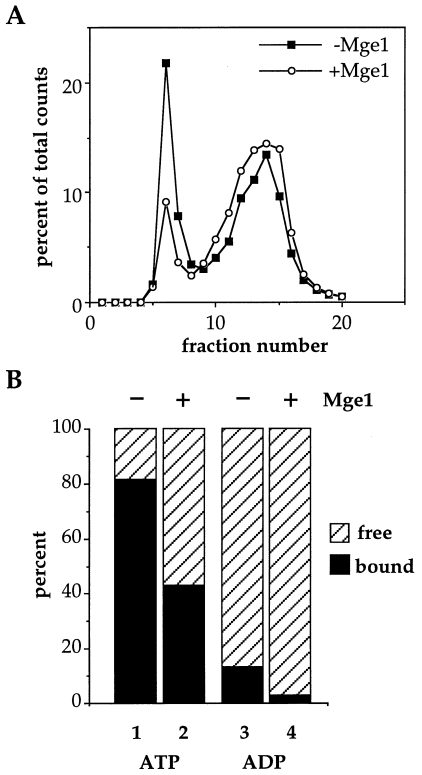

Figure 2. Mge1 releases both ATP and ADP from Ssc1. A, The Ssc1/ATP complex (~3 μM) was incubated with or without Mge1 (30 μM) at 30°C to achieve 50 to 60% conversion of ATP to ADP before loading onto a G-50 column. Aliquots were collected and counted. Percentage of total counts in each fraction was plotted. The first peak of radioactivity around fraction 6 corresponds to bound nucleotides whereas the second peak of radioactivity around fraction 14 corresponds to free nucleotides. B, Aliquots from peak fractions were mixed with stop solution immediately after emerging from the column and assayed for relative amount of ATP and ADP. Percentages of free and bound ATP and ADP were calculated from relative peak areas and ATP/ADP ratio in the peak fractions.

hydrolysis can occur. The rate of hydrolysis of the bound [α-^{32}P]ATP was not significantly affected by the addition of excess unlabeled ATP or ADP (Figure 1A), indicating that the bound ATP was stable in the time frame of the experiment. At longer time points, excess unlabeled ATP or ADP quenched the hydrolysis of bound [α-^{32}P]ATP, indicating that bound ATP was slowly released from Ssc1 (data not shown).

However, when Mge1 was added to the reaction, a significant drop in the rate of hydrolysis of [α-^{32}P]ATP was observed. Furthermore, when excess cold ATP or ADP was added together with Mge1, the hydrolysis of bound [α-^{32}P]ATP was almost completely quenched (Figure 1A). Together these results indicate that addition of Mge1 causes release of bound ATP from Ssc1. In the presence of excess cold ATP or ADP, the released [α-^{32}P]ATP was rarely rebound thus the hydrolysis of [α-^{32}P]ATP was almost totally quenched. In the absence of excess cold competitors, the released [α-^{32}P]ATP could be rebound by Ssc1, but this release and rebinding would slow down the rate of hydrolysis. If this hypothesis is true, one would predict that if the concentration of Mge1 wasn't already saturating, addition of more Mge1 would decrease the rate of hydrolysis further. Such a decrease was observed when the ratio of Mge1:Ssc1 was increased from 2:1 to 10:1 (Figure 1B).

To test more directly the effect of Mge1 on the release of bound nucleotide from Ssc1, isolated Ssc1/ATP complex was incubated at 30°C in the absence or presence of Mge1 to achieve 50 to 60% conversion of [α-^{32}P]ATP to [α-^{32}P]ADP. The reaction mixture was again subjected to size exclusion chromatography to monitor the release of bound ATP/ADP. Although a significant amount of radioactivity remained associated with Ssc1 in the absence of Mge1, a peak of free nucleotides was also observed (Figure 2A). Analysis of the peak fractions revealed that 81% of total ATP remained bound to Ssc1, whereas only 13% of total ADP was still bound to Ssc1. The free nucleotide peak contains the remaining 19% of ATP and 87% of

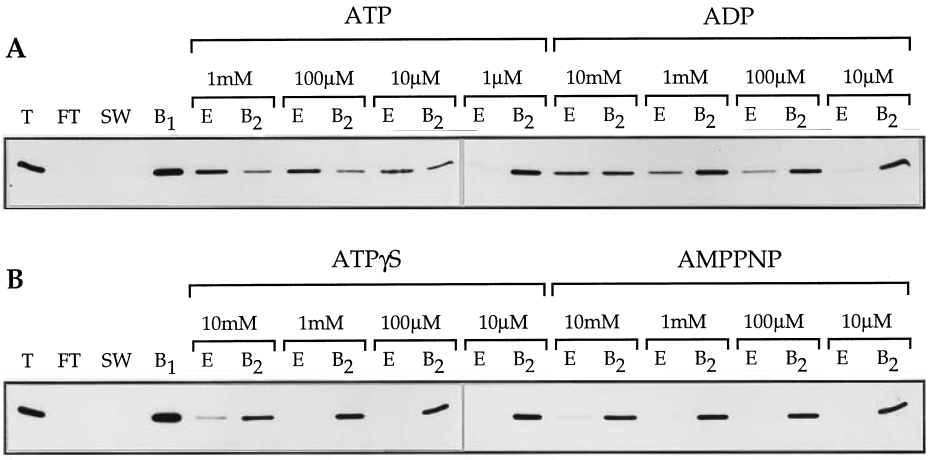

Figure 3. Effects of adenine nucleotides on the stability of the Ssc1/Mge1 complex. GST-Ssc1 fusion protein was immobilized onto glutathione agarose beads and purified Mge1 (T) was mixed with the beads. After incubation at 4°C for 30 minutes the unbound fraction was collected (FT). The beads were washed with buffer containing 1 M NaCl (SW), and a sample of the beads was taken (B_1). The beads were then eluted with the indicated concentrations of adenine nucleotides (E) and a sample of the beads after elution was taken (B_2). Immunoblots with anti-Mge1 antibody are shown.

ADP (Figure 2B, lane 1 and lane 3). This distribution of nucleotide suggests that ADP is preferably released from Ssc1, and ATP is relatively stably bound to Ssc1 during the time required to collect the bound fractions (~eight minutes at 4°C). This stability of bound ATP is consistent with the results shown in Figure 1A, as excess unlabeled ATP did not quench the hydrolysis of prebound [α-^{32}P]ATP. However, when Mge1 was added, a significant drop in the amount of radioactivity associated with Ssc1 was observed (Figure 2A), supporting the idea that Mge1 stimulates the release of nucleotides from Ssc1. Furthermore, in the presence of Mge1, the fraction of total ATP remaining associated with Ssc1 dropped from 81 to 47% (Figure 2B, lane 1 *versus* lane 2), whereas bound ADP dropped from 13 to only 3% (Figure 2B, lane 3 *versus* lane 4). This result indicates that Mge1 promotes the release of both ATP and ADP from Ssc1, and thus functions as a nucleotide release factor for Ssc1.

Mge1 can stably associate with Ssc1

Since Mge1 could function as a nucleotide release factor for Ssc1, we asked whether interaction between the two proteins could be detected directly. We took advantage of the fact that the GST-Ssc1 fusion protein, which we constructed for purification of Ssc1 (see Materials and Methods), could be immobilized onto glutathione agarose beads. After binding of GST-Ssc1 fusion protein to the glutathione affinity beads, purified Mge1 was mixed with the immobilized Ssc1. As shown in Figure 3A, essentially all the Mge1 was retained on the beads. In the absence of the GST-Ssc1 fusion protein, no retention of Mge1 on the beads was observed, indicating that the interaction with the GST-Ssc1 fusion protein was specific. This experiment was performed with sub-stoichiometric levels of Mge1, with the Ssc1:Mge1 ratio being 10:1. Control experiments showed that when excess Mge1 was added, the immobilized GST-Ssc1 fusion protein was capable of retaining approximately an equal molar amount of Mge1 (data not shown).

To test the stability of the Ssc1:Mge1 interaction, the beads were then washed extensively with 1 M NaCl. No Mge1 was detected in the salt wash fraction, indicating that the interaction is stable in the presence of high concentrations of salt. The susceptibility of the Mge1:Ssc1 interaction to nucleotides was also tested. Incubation in the presence of 1 mM ATP resulted in the release of the majority of the bound Mge1. The portion of Mge1 that remained in the beads fraction was probably due to incomplete separation by batch elution, since it was significantly diminished by more extensive washes with ATP (data not shown). As little as 10 μM ATP was effective in releasing Mge1 from Ssc1. ADP was much less effective in releasing Mge1 from Ssc1, requiring a concentration of 10 mM for significant release of Mge1. This release by high concentrations of ADP is not due to minor ATP contamination since pretreating the ADP with hexokinase had no effect on the release of Mge1 by ADP (data not shown). ATPγS and AMPPNP were not able to effectively release Mge1 from Ssc1 (Figure 3B). The faint bands that appeared in the 10 mM elution lanes could be attributed to ATP contamination in the commercially available ATPγS and AMPPNP (Ahsen *et al.*, 1995; Horst *et al.*, 1996). The failure of these ATP analogs to release Mge1 suggests that either the analogs do not bind to Ssc1 because of lower affinities as reported for Hsc70 (Gao *et al.*, 1994), or the binding of the analogs is different from the binding of ATP, as reported for DnaK (Liberek

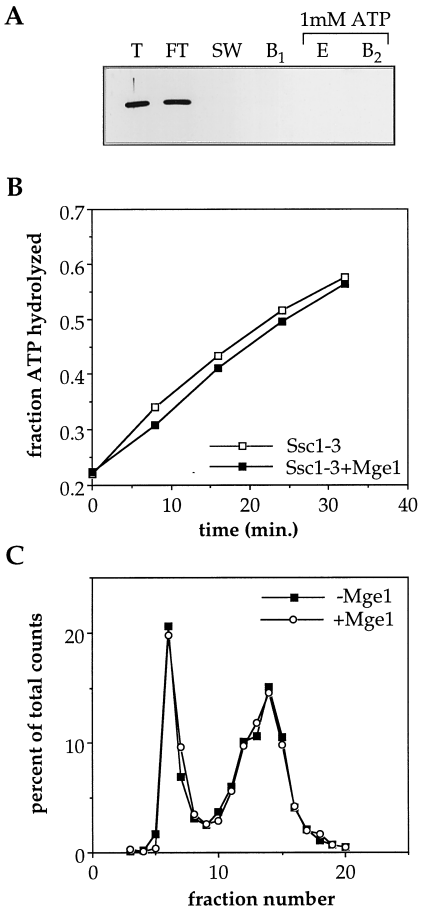

Figure 4. Ssc1-3 is defective in interaction with Mge1. A, GST-Ssc1-3 fusion protein was immobilized onto glutathione agarose beads and purified Mge1 was mixed with the beads. The beads were extensively washed and eluted with 1 mM ATP as described in Materials and Methods. T, Total amount of Mge1 loaded onto the beads; FT, flow through fraction; SW, salt wash with 1 M NaCl in buffer D; B_1, beads before elution with adenine nucleotides; E, Mge1 eluted with adenine nucleotides; B_2, beads after elution with adenine nucleotides. B, The Ssc1-3/ATP complex (~2 μM) was incubated at 30°C with or without 20 μM Mge1. Aliquots were withdrawn at the indicated time points and the fraction of ATP converted to ADP determined as described in Materials and Methods. C, The Ssc1-3/ATP complex (~3 μM) was incubated with or without Mge1 (30 μM) at 30°C to achieve 50 to 60% conversion of ATP to ADP before loading onto a G-50 column. Aliquots were collected and counted. Percentage of total counts in each fraction was plotted.

et al., 1991b; Palleros *et al.*, 1993) and Hsc70 (Ha & McKay, 1995)

Ssc1-3 is defective in interaction with Mge1

Previously it was reported that, although Mge1 could be co-immunoprecipitated with wild-type Ssc1 from isolated mitochondria, it could not be co-immunoprecipitated with Ssc1-3, a temperature-sensitive mutant of Ssc1 (Voos *et al.*, 1994). This lack of co-immunoprecipitation suggests that Ssc1-3 is defective in stable binding of Mge1. We investigated this interaction further in our assay systems, using purified components. As shown in Figure 4A, Mge1 was not retained when GST-Ssc1-3 protein was immobilized on the glutathione affinity matrix, indicating that indeed Ssc1-3 could not stably bind Mge1.

We then tested whether Ssc1-3 could functionally interact with Mge1, even though it did not stably bind to it. A Ssc1-3/ATP complex was isolated and tested in the single turnover assay. In contrast to the inhibition of the hydrolysis of [α-^{32}P]ATP observed with wild-type Ssc1, addition of Mge1 had no effect on the hydrolysis of [α-^{32}P]ATP by Ssc1-3 (Figure 4B). Also, addition of Mge1 to the Ssc1-3/ATP complex did not cause release of bound nucleotides from Ssc1-3 (Figure 4C). This inability of Mge1 to affect hydrolysis and release of prebound nucleotides indicates that Ssc1-3 neither stably binds nor functionally interacts with Mge1.

The role of a conserved loop in interaction of Ssc1 with Mge1

A previous study has identified a conserved loop on the surface of DnaK which is important for interaction with GrpE (Buchberger *et al.*, 1994a). The alteration in Ssc1-3, G79S, lies very close to the analogous loop, E56 to R62, on the modeled Ssc1 tertiary structure (the alpha-carbon atom of G79 is approximately 10 Å away from the alpha-carbon atom of E59). This close proximity raises the possibility that this corresponding loop of Ssc1 is important for interaction with Mge1. To test this hypothesis, we constructed several mutants in this region including ones analogous to those which had been tested in *E. coli*. Δ56-61, corresponding to Δ28-33 in DnaK, removes the conserved loop, and G60D, corresponding to G32D in DnaK, changes a highly conserved residue within the loop (Buchberger *et al.*, 1994a). Additional mutations were also constructed which result in alterations in E59, a residue conserved between DnaK and Ssc1 as well as several other bacterial and mitochondrial Hsp70s (Figure 5A, and Boorstein *et al.*, 1994).

The phenotype of these mutants was determined by testing the ability of a *TRP1*-based plasmid containing the mutant *SSC1* gene to rescue growth of the strain JD100 on media containing 5-FOA. The chromosomal copy of *SSC1* is disrupted in the strain JD100, and the viability of the strain is maintained by a *URA3*-based plasmid containing a functional *SSC1* gene. 5-FOA is converted to a toxic metabolite by cells expressing the *URA3* gene, thus only cells which have lost the *URA3*-based plasmid are viable on medium containing 5-FOA. Under these conditions, the only copy of *SSC1* left in the cell is the mutant *SSC1* on the *TRP1*-based plasmid. The growth phenotype of the resulting strain thus reflects the phenotype of

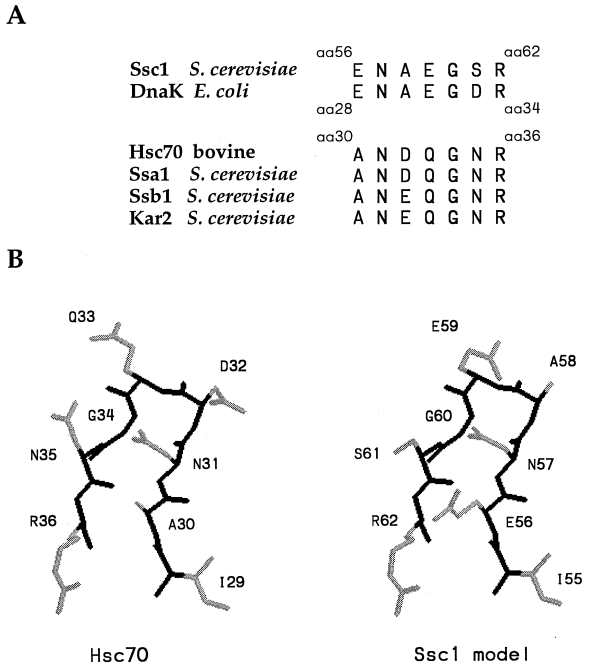

Figure 5. A model of the conserved loop structure in Ssc1. A, Sequence alignment of the loop region from several Hsp70s. B, Tertiary structures of the loop region of Hsc70 and Ssc1. Data for the Hsc70 structure were obtained from the Brookhaven Data Bank. Data for the proposed Ssc1 structure were obtained from Gene Crunch, a Yeast Genome Analysis on a Silicon Graphics Supercomputer (http://genecrunch.sgi.com). The structures were generated using RasMol, a program to display protein tertiary structures on Macintosh.

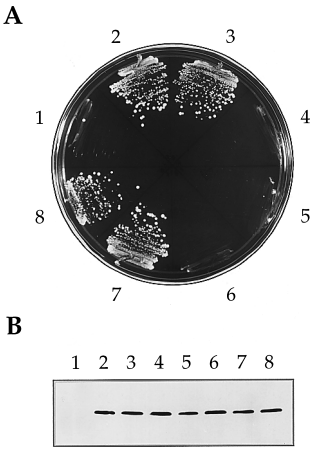

Figure 6. Phenotypes of *SSC1* mutants. A, Strain JD100 was transformed with pRS314, pRS314-SSC1, or mutant *SSC1* genes carried on pRS314. Transformants were streaked on plates containing 5-FOA and incubated at 30°C for three days. B, The same transformants were grown in media lacking tryptophan, uracil and leucine at 30°C overnight. Cells were collected and boiled in SDS-PAGE sample buffer before loading onto a SDS-10% PAGE gel. An immunoblot using anti-Ssc1 antibody is shown. 1, Vector only; 2, wild-type *SSC1*; 3, *ssc1-3*; 4, *Δ56-61*; 5, *G60D*; 6, *E59K*; 7, *E59A*; 8, *E59D*.

the mutant *SSC1*. Three of the mutants, *Δ56-61, G60D* and *E59K*, had a null phenotype, as cells expressing the mutant gene were unable to form colonies at 30°C and 37°C (Figure 6A, and data not shown). Two of the mutants, *E59A* and *E59D*, permitted wild-type growth rates.

To assure that the null phenotype observed was not due to instability of the mutant Ssc1, the level of mutant protein in cells was assessed. In the strain JD100, the functional *SSC1* protein encoded by the *URA3*-based plasmid lacks the C-terminal 21 amino acids. Since the Ssc1 antibody is raised against a peptide corresponding to the sequence of the last 14 amino acids of Ssc1, an immunoblot using this antibody does not detect the truncated yet functional Ssc1, but rather only the mutant *SSC1* protein. As shown in Figure 6B, all mutant *SSC1* proteins were stable, and steady state levels of mutant proteins were indistinguishable from wild-type Ssc1.

To analyze the interaction between the mutant *SSC1* proteins and Mge1, GST-Ssc1 mutant proteins were immobilized and tested in the stable binding assay described above. As shown in Figure 7, Δ56-61, which lacks the proposed loop structure, failed to stably bind Mge1, as expected. Interestingly, the point mutants of the loop affected the stable binding of Mge1 little if at all. E59A and E59D, which were functional *in vivo*, interacted with Mge1 indistinguishably from wild-type Ssc1. G60D and E59K, which did not provide Ssc1 function *in vivo*, were able to bind Mge1. A

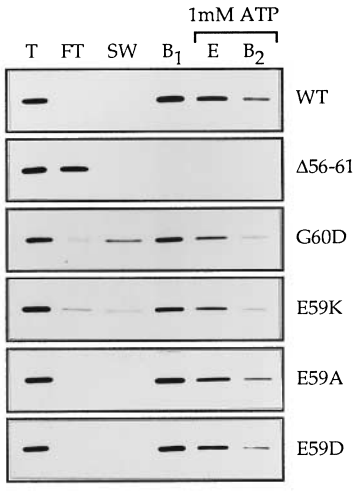

Figure 7. Effects of loop mutations of Ssc1 on the stable binding of Mge1. GST-Ssc1 mutant fusion proteins were immobilized onto glutathione agarose beads and purified Mge1 was mixed with the beads. The beads were extensively washed and eluted with 1 mM ATP as described in Materials and Methods. T, Total amount of Mge1 loaded onto the beads; FT, flow through fraction; SW, salt wash with 1 M NaCl in buffer D; B_1, beads before elution with adenine nucleotides; E, Mge1 eluted with adenine nucleotides; B_2, beads after elution with adenine nucleotides.

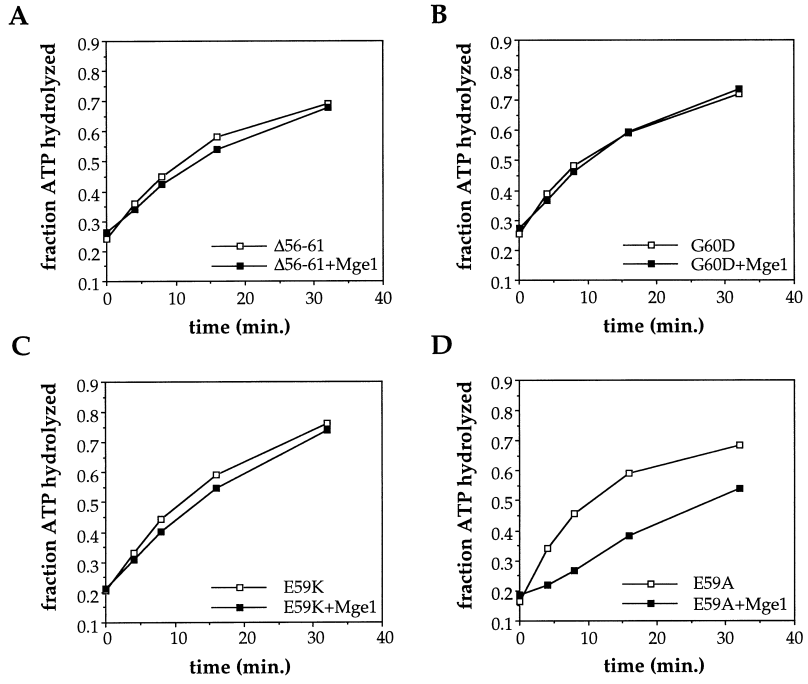

Figure 8. Effects of loop mutations of Ssc1 on its functional interaction with Mge1. Each mutant Ssc1/ATP complex (~2 μM) was incubated at 30°C with or without 20 μM Mge1. Aliquots were withdrawn at the indicated time points and the fraction of ATP converted to ADP determined as described in Materials and Methods.

fraction of the Mge1 bound to G60D eluted with the 1 M salt wash, suggesting that the ionic interactions between the two proteins are altered by the mutation. When the salt concentration in the wash was lowered to 150 mM, which is more physiological, all of the Mge1 remained bound to the GST-Ssc1 mutant protein (data not shown), suggesting that the G60D is able to bind Mge1 *in vivo*. The portion of Mge1 that remained bound to G60D after the 1 M salt wash was eluted with 1 mM ATP, indicating that the interaction is still sensitive to nucleotides.

To test the functional interaction between the mutant proteins and Mge1, we purified the mutant proteins and tested the effect of Mge1 in single turnover assays, as an indirect measure of the ability of Mge1 to stimulate nucleotide release. Mge1 did not affect the hydrolysis of [α-^{32}P]ATP by Δ56-61 (Figure 8A), as expected since the two proteins did not form a stable complex in the stable binding assay. However, Mge1 did affect the hydrolysis of [α-^{32}P]ATP bound by E59A (Figure 8D) and E59D (data not shown), indicating that these changes do not interfere with the Ssc1:Mge1 interaction. Interestingly, Mge1 had little effect if at all on the hydrolysis of [α-^{32}P]ATP by G60D and E59K (Figure 8B and C), which bound Mge1 in the stable binding assay. This failure to inhibit ATP hydrolysis suggests that, although these two mutant *SSC1* proteins bind Mge1, this binding does not affect the release of ATP from the mutant *SSC1* proteins.

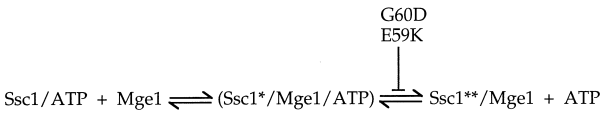

Figure 9. Proposed interactions among Ssc1, Mge1 and ATP. The proposed blocking site for the G60D and E59K mutations are also shown. Ssc1 and Ssc1**, the proposed different conformations of the *SSC1* protein; (Ssc1*/Mge1/ATP), transient intermediate.

Discussion

Based on its sequence similarity to GrpE and its interaction with Ssc1 in extracts of mitochondria, Mge1 has been proposed to function as a nucleotide release factor for Ssc1 (Laloraya *et al.*, 1995; Westerman *et al.*, 1995; Nakai *et al.*, 1994). The experiments reported here demonstrate that Mge1 is indeed a nucleotide release factor. In a concentration dependent manner the apparent rate of hydrolysis of [α-^{32}P]ATP bound by Ssc1 is inhibited by Mge1, due to the stimulation of ATP release by Mge1. This inhibition is nearly complete in the presence of excess unlabeled nucleotide because the released radiolabeled ATP must compete for rebinding with the excess unlabeled nucleotide. Analysis of the radiolabeled nucleotides remaining bound to and released from Ssc1 demonstrates that the release of both ADP and ATP is stimulated by Mge1.

Both Ssc1 and Mge1 are required for the proper translocation of cytosolic precursor proteins into mitochondria, as conditional mutants of either cause a delay or block of import of precursor proteins. How might Ssc1 and Mge1 function together in this process? It has been shown that the binding of Ssc1 to precursor proteins in transit across the mitochondrial membranes is crucial for the import process (Kang *et al.*, 1990; Gambill *et al.*, 1993; Ungermann *et al.*, 1994). Subsequent release of

imported proteins from Ssc1 allows the proper folding of these proteins, and allows the freed Ssc1 to function again at the import site. It has been proposed that the ATP-bound form of Hsp70, which binds peptides much faster, but also releases them faster than the ADP-bound form, is the Hsp70 form which actively binds polypeptides (Greene *et al*., 1995; McCarty *et al*., 1995). Subsequent ATP hydrolysis converts Hsp70 to an ADP-bound form, which has a higher affinity for peptides, thus stabilizing the interaction. The efficient release of peptides from Hsp70 requires the release of bound ADP and subsequent binding of ATP. ADP release is stimulated by nucleotide release factors such as Mge1. Because normally the ATP concentration is higher than the ADP concentration in mitochondria, Ssc1 is more likely to bind ATP than ADP when bound nucleotide is released upon Mge1 binding. Therefore by facilitating nucleotide release, Mge1 helps to more efficiently cycle Ssc1 between the ADP-bound form and the ATP-bound form, thus facilitating cycles of polypeptide binding and release.

Similar to the interaction of GrpE and DnaK, Mge1 binds Ssc1 stably. This interaction is not disrupted by 1 M NaCl, but is sensitive to the addition of ATP. The Ssc1/Mge1 complex is disrupted at an ATP concentration as low as 10 μM. This is in excellent agreement with results obtained in isolated mitochondria, where it has been shown that Mge1 is released from Ssc1 at 10 μM ATP but not 1 μM ATP (Bolliger *et al*., 1994). ADP is much less efficient in disrupting this complex in our binding assay; 10 mM ADP was required for substantial release of Mge1. Since Mge1 stimulates the release of both ATP and ADP from Ssc1, and only ATP efficiently disrupts the Ssc1/Mge1 complex, in essence Mge1 shifts the equilibrium to favor an ATP-bound state of Ssc1. Thus Mge1 can be thought of as a nucleotide exchange factor, since the net effect is conversion of Ssc1 from an ADP-bound state to an ATP-bound state.

Based on analogy to the bovine Hsc70 structure, a small loop stabilized by hydrogen bonds protrudes from the surface of the ATPase domain of both DnaK and Ssc1 (Figure 5B, see also Buchberger *et al*., 1994a). Deletion of the loop eliminates the ability of Mge1 to interact with Ssc1, as well as GrpE to interact with DnaK. In the case of DnaK756, a single amino acid change within the loop, G32D, prevents a stable interaction with GrpE. However, the analogous alteration in Ssc1, G60D, allowed stable binding of Mge1. This apparent discrepancy between DnaK and Ssc1 could be due to experimental differences. The DnaK:GrpE interaction was assessed by non-denaturing PAGE (Buchberger *et al*., 1994a), whereas the Ssc1:Mge1 interaction was assessed by the ability of Mge1 to stably associate with the immobilized Ssc1.

Whereas Mge1 bound G60D, it failed to stimulate nucleotide release. In single turnover experiments, addition of Mge1 had no effect on the hydrolysis of ATP bound by G60D. Two explanations for the failure of Mge1 to stimulate ATP release from G60D are: (1) Mge1 fails to bind G60D when it is in the ATP-bound form; (2) Mge1 binds G60D/ATP, but this binding does not stimulate the release of bound ATP. Since even wild-type Ssc1 does not bind Mge1 stably in the presence of ATP, we can not test whether G60D binds Mge1 when it is in the ATP-bound form. However, we were able to test whether G60D binds Mge1 in the ADP-bound form. While other Hsp70s have been shown to retain ADP during purification (Gao *et al*., 1994; Wei & Hendershot, 1995), we were not certain whether the immobilized GST-Ssc1 protein used in the stable binding assay contained bound ADP. However, both wild-type Ssc1 and G60D bound Mge1 stably in the presence of 10 μM ADP (data not shown), a concentration insufficient to affect Mge1 release from Ssc1, but at least tenfold above the K_d for ADP for several Hsp70s analyzed (Gao *et al*., 1993; Ha & McKay, 1994; Palleros *et al*., 1993; Schmid *et al*., 1985; Wang & Lee, 1993).

Mge1 must bind both the ATP-bound form and the ADP-bound form of wild-type Ssc1 since it releases both ATP and ADP from Ssc1. This stimulation of release of both ATP and ADP by Mge1 suggests that the Mge1-binding interface of Ssc1 is similar in both the ATP-bound and the ADP-bound form. Based on the ability of the ADP-bound form of G60D to bind Mge1, it is unlikely that the ATP-bound form of G60D is incompetent in Mge1 binding. Therefore we propose that the G60D mutant is defective in Mge1-induced nucleotide release rather than binding of Mge1.

Similar results were obtained for a mutant causing an alteration at an adjacent amino acid, E59K. Interestingly, E59A and E59D, two other changes at residue E59, did not affect the physical or functional interaction between Ssc1 and Mge1 in our assays. This residue is conserved between DnaK and Ssc1 (Figure 5A, see also Boorstein *et al*., 1994), and has been proposed to be involved in the DnaK:GrpE interaction (Buchberger *et al*., 1994a). However, the fact that E59 can be changed to an alanine residue without affecting the Ssc1:Mge1 interaction argues that the negative charge of E59 is not critical for this interaction.

It is intriguing that Mge1 failed to release ATP from the two mutant proteins, G60D and E59K, yet ATP could cause the release of Mge1. The following mechanism may explain this apparent discrepancy. Considering the equation shown in Figure 9, the release of ATP by Mge1 and the release of Mge1 by ATP are opposite directions of the same reaction, presumably *via* a common transient tertiary complex of Ssc1/Mge1/ATP. We propose that as far as the interactions among Ssc1, Mge1 and ATP are concerned, Ssc1 can exist in at least two conformations, one with ATP bound that has a low affinity for Mge1 (Ssc1 in Figure 9), the

other binds Mge1 tightly but has a low affinity for ATP (Ssc1** in Figure 9). Binding of ATP to Ssc1 will induce a conformational change in Ssc1 which results in Mge1 release; binding of Mge1 will also change the conformation of Ssc1, causing ATP release. In the case of DnaK, ATP-induced conformational changes have been well documented (Banecki & Zylicz, 1996; Liberek *et al*., 1991b; Palleros *et al*., 1992); fluorescence measurements suggest that GrpE also induces a conformational change in DnaK (Reid & Fink, 1996).

According to this model, the relative concentrations of ATP and Mge1 available will determine the favorable conformation of Ssc1 in our *in vitro* assays. In single turnover experiments, a large excess of Mge1 was present, thus ATP was released from Ssc1. On the other hand, in stable binding experiments, when ATP elution was performed, ATP was present in excess, thus Mge1 was released from Ssc1. The two mutations on the loop, G60D and E59K, may only significantly affect one direction of the equilibrium, namely, the release of ATP by Mge1, but not the other direction, the release of Mge1 by ATP. Thus the binding of ATP to these two mutant *SSC1* proteins causes the conformational change necessary for releasing Mge1, but the binding of Mge1 to the mutant *SSC1* proteins does not cause the conformational change in the mutant *SSC1* proteins required for nucleotide release.

These defects point to a more sophisticated role of the E56-R62 loop in the Ssc1:Mge1 interaction. We propose that this loop, as well as its surrounding areas on the tertiary structure, are important for forming the binding interface for Mge1. Ssc1-3 may significantly affect this binding interface, thus blocking the physical and functional interactions between the two proteins. The deletion of the loop may have a similar effect in disrupting the Ssc1:Mge1 interaction. However, while playing a role in Mge1-Ssc1 association, this loop is crucial for the ability of Mge1 to induce a conformational change of Ssc1, which leads to the release of bound nucleotide. One possibility is that upon Mge1 binding, the conformation of the loop changes, which transmits conformational changes to the nucleotide binding site, causing nucleotide release. G60D and E59K may alter the conformation of the loop in a way that compromises the transmission of these changes, thus affecting nucleotide release without significantly affecting the binding of Mge1 to Ssc1. This is the first demonstration of the separation of Mge1 binding and nucleotide release induced by binding of Mge1. Since no analogous mutants have been found in DnaK, further analysis of these two mutants should shed more light on the mechanism of nucleotide release from Hsp70s induced by GrpE/Mge1.

Materials and Methods

Bacterial and yeast strains

PK101: F-, Kan^R, ΔdnaKJ. Chromosomal copy of *dnaK* and part of *dnaJ* are deleted (Kang & Craig, 1990). This strain was used for expression of GST-Mge1 fusion protein.

BJ3497: *pep4::HIS3 ura3-52 his*Δ*200*. This strain is defective in Proteinase A (Jones, 1991), and was used for expression of GST-Ssc1 fusion proteins.

JD100: *lys2 ura3-52* Δ*trp1 leu2-3,112 ssc1-1(LEU2)*. The chromosomal copy of the *SSC1* gene is disrupted with *LEU2*; Ssc1 function is provided by the truncated Ssc1 encoded on the plasmid pJD1. This truncated *SSC1* protein lacks the last 21 amino acids and is functional, but is not detected by a Ssc1 antibody which is raised against a peptide corresponding to the last 14 amino acids of Ssc1. This strain was used to check phenotypes as well as *SSC1* protein levels of various *SSC1* mutants.

Plasmids

pGEXKT-MGE1: a *Bam*HI site was generated using PCR at nucleotides 127 to 132 (1 being the A in ATG) of *MGE1* using the primer 5′-CCCATGGGATCCGATGAAGCCAAAAGTGAAGAATCC-3′. The PCR-generated fragment was either sequenced or replaced with a wild-type fragment to ensure that no PCR-induced mutation was present. The putative mature *MGE1* protein was fused to GST by cloning the PCR fragment into pGEX-KT (Hakes & Dixon, 1992) as a *Bam*HI-*Xho*I fragment.

pRD56CS-SSC1: a *Bam*HI site was generated using PCR at nucleotides 70 to 75 of *SSC1* (1 being the A in ATG) using the primer 5′-ACACGTTTGGGATCCACCAA-3′. The PCR-generated fragment was either sequenced or replaced with a wild-type fragment to ensure that no PCR-induced mutation was present. The mature *SSC1* protein was fused to GST by cloning the PCR fragment into pRD56CS (pRD56 (Park *et al*., 1993) with *Cla*I and *Sal*I sites filled in with Klenow) as a *Bam*HI–*Eco*RI fragment. The encoded mature *SSC1* protein has a glycine residue instead of a glutamine residue at the N terminus.

pJD1: a *Xho*I site was generated using PCR at nucleotides 1900 to 1905 of *SSC1* (1 being the A in ATG) using the primer 5′-AATTATACAAGCTCGAGTCTAACAA-3′. The *Xho*I site was then filled in with Klenow to generate a stop codon downstream. The encoded *SSC1* protein has the last 21 amino acids replaced with LDRV (Kang, 1991). This truncated *SSC1* was cloned into pRS316 as a *Xba*I–*Eco*RI fragment.

pRS314-SSC1: wild-type *SSC1* was cloned into pRS314 as a *Pst*I–*Bam*HI fragment.

Protein expression and purification

Yeast strain BJ3497 harboring the expression plasmid pRD56CS-SSC1 was grown for two days at 30°C in 50 ml of media lacking uracil with galactose as the carbon source. This culture was then inoculated into one liter of YPGal media and grown overnight at 30°C. Cells were harvested and resuspended in 12 ml of buffer A (16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 150 mM NaCl) containing 1% (v/v) Triton X-100. Cells were disrupted with a French Pressure Cell (SLM-Aminco, Urbana, IL), and spun at 20,000 *g* for 15 minutes. The soluble extract

was incubated with 12.5 ml of glutathione agarose beads (prepared as in Lew *et al.*, 1991) for one hour at 4°C. The beads were washed extensively with buffer A containing 1% Triton X-100, buffer A containing 1 M NaCl, buffer A, buffer B (50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2.5 mM $CaCl_2$), and then 50 units of thrombin (Sigma T-3010) were added. After three hours of cleavage at 4°C, the cleavage product was collected and the beads were washed twice to collect more cleavage product trapped in the beads. The pooled cleavage product was concentrated in a Centriprep-10 (Amicon, Danvers, MA), adjusted to 10% (v/v) glycerol, aliquoted and stored frozen at −70°C. The protein preparation was greater than 90% pure as judged from Coomassie blue staining.

E. coli strain PK101 harboring the expression plasmid pGEXKT-MGE1 was grown to mid log phase at 30°C and induced with 0.1 mM IPTG for three hours. Cells were harvested and processed essentially the same as above, except the thrombin cleavage was allowed to proceed for 48 to 72 hours at 4°C. The protein preparation was greater than 95% pure as judged from Coomassie blue staining.

Mutant *SSC1* proteins were purified the same way as the wild-type Ssc1.

Complex formation and single turnover experiments

Ssc1 (50 μg) was incubated with [α-^{32}P]ATP (10 μCi, Dupont NEG-003H, 3000 Ci/mmol) in buffer C (50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 2 mM DTT) containing 25 μM ATP at 30°C for 15 minutes. The reaction mixture was chilled on ice and immediately loaded onto a 2 ml G-50 (superfine) column, pre-equilibrated with buffer C, at 4°C. 60 μl fractions were collected. Fractions were monitored for radioactivity with a Geiger counter, and the first peak of radioactivity corresponding to the Ssc1/ATP complex was pooled, adjusted to 10% glycerol, aliquoted and stored frozen at −70°C.

For single turnover experiments, 10 μl Ssc1/ATP complex was quickly thawed and added to 10 μl buffer C containing various factors, and incubated at 30°C. At the indicated time points, 3 μl of the reaction mixture was withdrawn and mixed with 1 μl of stop solution containing 4 M formic acid, 2 M LiCl and 36 mM ATP. This mixture was then spotted onto a PEI-cellulose TLC plate (Sigma Z12,288-2) and developed in 1 M formic acid and 0.5 M LiCl. The TLC plate was dried and exposed to a phosphorimager screen and data quantified on a phosphorimager system (Molecular Dynamics, Sunnyvale, CA). The data were plotted using Cricket Graph. All single turnover experiments shown were performed at least three times with similar results; representative results from one experiment are shown in each case.

In the experiment where the Ssc1/ATP complex was refractionated on G-50, the Ssc1/ATP complex was quickly thawed and incubated with or without Mge1 at 30°C for short periods of time to achieve 50 to 60% conversion of [α-^{32}P]ATP to [α-^{32}P]ADP. The reaction mix was then chilled on ice and immediately loaded onto a 2 ml G-50 (superfine) column pre-equilibrated with buffer C at 4°C. 120 μl fractions were collected. Aliquots from peak fractions were immediately mixed with stop solution and later developed on PEI-cellulose TLC plates to determine the relative amount of ATP and ADP in each fraction. An amount (50 μl) of each fraction was then counted in a liquid scintillation counter to determine the amount of radioactivity associated with each fraction. The amount of ATP and ADP in the bound *versus* free peak was calculated from relative peak areas and relative amount of ATP and ADP in each peak fraction. At least three independent experiments were performed with similar results; representative results from one experiment are shown in each case.

Stable binding of Mge1 to Ssc1

GST-Ssc1 fusion protein was immobilized on glutathione agarose affinity beads as described above. The beads were washed extensively with buffer A containing 1% Triton X-100, buffer A containing 1 M NaCl, buffer A, and buffer D (25 mM Hepes-KOH, pH 7.4, 50 mM KCl, 10% glycerol, 1 mM EDTA). An equal volume of 0.1 μM Mge1 was added to the beads and incubated at 4°C for 30 minutes. The beads were then washed with buffer D, buffer D containing 1 M NaCl, buffer D, and eluted with buffer D containing 10 mM $MgCl_2$ and the indicated amount of ATP, ADP or various ATP analogs (ATP, Sigma A2383; ADP, Sigma A5136; AMPPNP, Sigma A2647; ATPγS, Sigma A1388). Samples were collected at various stages and separated by SDS- 12% PAGE, blotted and probed with antibodies against Mge1. ECL westerns (Amersham, Arlington Heights, IL) were performed according to the manufacturer's suggestions.

Site-directed mutagenesis of *SSC1*

Most mutants were generated using a standard two-step PCR procedure (Cormack, 1994). The PCR fragment was sequenced to ensure the desired mutation was present and no other PCR induced changes were present. For the loop deletion mutant, a linker to allow incorporation of a glycine residue and an alanine residue was added, as in the case of the corresponding DnaK mutant (Buchberger *et al.*, 1994a). Primers used to generate the mutants are as follows: Δ56-61, 5′-CAAAAATTATTGCTGGTAGAACTACTCCTTCTGTAG-3′ and 5′-TTCTACCAGCAATAATTTTTGGAACTTTACCT-3′; G60D, 5′-AAACGCCGAAGATTCCAGAACT-3′ and 5′-AGTTCTGGAATCTTCGGCGTTT-3′; E59K, 5′-TGAAAACGCCAAGGGTTCCAGAA-3′ and 5′-GTTCTGGAACCCTTGGCGTTTTC-3′. Mutants E59A and E59D were generated using a standard M13 mutagenesis procedure (Kunkel, 1985). The degenerate primer 5′-AGGAGTAGTWSYGGAACCWKCGGCGTTTTC-3′, where W is a A or T; S is a G or C; Y is a C or T; and K is a G or T, was used.

Mutants were sequenced to verify the presence of the desired mutation and the absence of other mutations. The mutant gene was then subcloned into pRS314-SSC1 for testing the phenotype and protein expression level, and into pRD56CS-SSC1 for testing Mge1 binding. The mutant proteins were purified as described above for use in single turnover assays.

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References

Ahsen, O. v., Voos, W., Henninger, H. & Pfanner, N. (1995). The mitochondrial protein import machinery. Role of ATP in dissociation of the Hsp70/Mim44 complex. *J. Biol. Chem.* **270**, 29848–29853.

Banecki, B. & Zylicz, M. (1996). Real time kinetics of the DnaK/DnaJ/GrpE molecular chaperone machine action. *J. Biol. Chem.* **271**, 6137–6143.

Blond-Elguindi, S., Cwirla, S., Dower, W., Lipshutz, R., Sprang, S., Sambrook, J. & Gething, M. J. (1993). Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. *Cell,* **75**, 717–728.

Bolliger, L., Deloche, O., Glick, B., Georgopoulos, C., Jeno, P., Kronidou, N., Horst, M., Morishima, N. & Schatz, G. (1994). A mitochondrial homolog of bacterial GrpE interacts with mitochondrial hsp70 and is essential for viability. *EMBO J.* **13**, 1998–2006.

Boorstein, W. R., Ziegelhoffer, T. & Craig, E. A. (1994). Molecular evolution of the HSP70 multigene family. *J. Mol. Evol.* **38**, 1–17.

Bork, P., Sander, C. & Valencia, A. (1992). An ATPase domain common to procaryotic cell cycle proteins, sugar kinases, actin and hsp70 heat shock proteins. *Proc. Natl Acad. Sci. USA,* **89**, 7290–7294.

Buchberger, A., Schroder, H., Buttner, M., Valencia, A. & Bukau, B. (1994a). A conserved loop in the ATPase domain of the DnaK chaperone is essential for stable binding of GrpE. *Nature: Struct. Biol.* **1**, 95–101.

Buchberger, A., Valencia, A., McMacken, R., Sander, C. & Bukau, B. (1994b). The chaperone function of DnaK requires the coupling of ATPase activity with substrate binding through residue E171. *EMBO J.* **13**, 1687–1695.

Buchberger, A., Theyssen, H., Schroder, H., McCarty, J. S., Virgallita, G., Milkereit, P., Reinstein, J. & Bukau, B. (1995). Nucleotide-induced conformational changes in the ATPase and substrate binding domains of the DnaK chaperone provide evidence for interdomain communication. *J. Biol. Chem.* **270**, 16903–16910.

Chappell, T. G., Konforti, B. B., Schmid, S. L. & Rothman, J. E. (1987). The ATPase core of a clathrin uncoating protein. *J. Biol. Chem.* **262**, 746–751.

Cormack, B. (1994). Introduction of a point mutation by sequential PCR steps. In *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K., eds.), vol. 1, pp. 8.5.7–8.5.9, John Wiley & Sons, New York.

Craig, E. A., Gambill, B. D. & Nelson, R. J. (1993). Heat shock proteins: molecular chaperones of protein biogenesis. *Microbiol. Rev.* **57**, 402–414.

Cyr, D. M., Langer, T. & Douglas, M. G. (1994). DnaJ-like proteins: molecular chaperones and specific regulators of Hsp70. *Trends Biochem. Sci.* **19**, 176–181.

Flaherty, K. M., DeLuca-Flaherty, C. & McKay, D. B. (1990). Three dimensional structure of the ATPase fragment of a 70k heat-shock cognate protein. *Nature,* **346**, 623–628.

Flaherty, K. M., McKay, D. B., Kabash, W. & Holmes, K. (1991). Similarity of the three-dimensional structure of actin and the ATPase fragment of a 70kDa heat shock cognate protein. *Proc. Natl Acad. Sci. USA,* **88**, 5041–5045.

Flynn, G. C., Chappell, T. G. & Rothman, J. E. (1989). Peptide binding and release by proteins implicated as catalysts of protein assembly. *Science,* **245**, 385–390.

Freeman, B., Myers, M., Schumacher, R. & Morimoto, R. (1995). Identification of a regulatory motif in Hsp70 that affects ATPase activity, substrate binding and interaction with HDJ-1. *EMBO J.* **14**, 2281–2292.

Gambill, B. D., Voos, W., Kang, P. J., Miao, B., Langer, T., Craig, E. A. & Pfanner, N. (1993). A dual role for mitochondrial heat shock protein 70 in membrane translocation of preproteins. *J. Cell Biol.* **123**, 109–117.

Gao, B., Emoto, Y., Greene, L. & Eisenberg, E. (1993). Nucleotide binding properties of bovine brain uncoating ATPase. *J. Biol. Chem.* **268**, 8507–8513.

Gao, B., Greene, L. & Eisenberg, E. (1994). Characterization of nucleotide-free uncoating ATPase and its binding to ATP, ADP, and ATP analogues. *Biochemistry,* **33**, 2048–2054.

Georgopoulos, C., Liberek, K., Zylicz, M. & Ang, D. (1994) . Properties of the heat shock proteins of *Escherichia coli* and the autoregulation of the heat shock response. In *The Biology of Heat Shock Proteins and Molecular Chaperones* (Morimoto, R. I., Tissieres, A. & Georgopoulos, C., eds.), pp. 209–250, Cold Spring Harbor Laboratory Press, Plainview, NY.

Gragerov, A., Zeng, L., Zhao, X., Burkholder, W. & Gottesman, M. E. (1994). Specificity of DnaK-peptide binding. *J. Mol. Biol.* **235**, 848–854.

Greene, L., Zinner, R., Naficy, S. & Eisenberg, E. (1995). Effect of nucleotide on the binding of peptides to 70-kDa heat shock proteins. *J. Biol. Chem.* **270**, 2967–2973.

Ha, J.-H. & McKay, D. (1994). ATPase kinetics of recombinant bovine 70kDa heat shock cognate protein and its amino-terminal ATPase domain. *Biochemistry,* **33**, 14625–14635.

Ha, J. H. & McKay, D. B. (1995). Kinetics of nucleotide-induced changes in the tryptophan fluorescence of the molecular chaperone Hsc70 and its subfragments suggest the ATP-induced conformational change follows initial ATP binding. *Biochemistry,* **34**, 11635–44.

Hakes, D. J. & Dixon, J. E. (1992). New vectors for high level expression of recombinant proteins in bacteria. *Anal. Biochem.* **202**, 293–298.

Hartl, F. U. (1996). Molecular chaperones in cellular protein folding. *Nature,* **381**, 571–580.

Horst, M., Oppliger, W., Feifel, B., Schatz, G. & Glick, B. (1996). The mitochondrial import motor: dissociation of mitochondrial hsp70 from its membrane anchor requires ATP binding rather than ATP hydrolysis. *Protein Sci.* **5**, 759–767.

Ikeda, E., Yoshida, S., Mitsuzawa, H., Uno, I. & Toh-e, A. (1994). *YGE1* is a yeast homologue of *Escherichia coli* grpE and is required for maintenance of mitochondrial function. *FEBS Letters,* **339**, 265–268.

Jones, E. W. (1991). Tackling the protease problem in *Saccharomyces cerevisiae*. *Methods Enzymol.* **194**, 428–453.

Jordan, R. & McMacken, R. (1995). Modulation of the ATPase activity of the molecular chaperone DnaK by peptides and the DnaJ and GrpE heat shock proteins. *J. Biol. Chem,* **270**, 4563–4569.

Kang, P. J. (1991). Functional studies of 70 kDa heat shock proteins in *E. coli* and yeast. Doctoral dissertation, University of Wisconsin-Madison, WI.

Kang, P. J. & Craig, E. A. (1990). Identification and characterization of a new *Escherichia coli* gene that is a dosage-dependent suppressor of a *dnaK* deletion mutation. *J. Bacteriol.* **172**, 2055–2064.

Kang, P. J., Ostermann, J., Shilling, J., Neupert, W., Craig, E. A. & Pfanner, N. (1990). Hsp70 in the mitochondrial matrix is required for translocation and folding of precursor proteins. *Nature,* **348**, 137–143.

Kunkel, T. A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl Acad. Sci. USA,* **82**, 488–492.

Laloraya, S., Gambill, B. D. & Craig, E. A. (1994). A role for a eukaryotic GrpE-related protein, Mge1p, in protein translocation. *Proc. Natl Acad. Sci. USA,* **91**, 6481–6485.

Laloraya, S., Dekker, P., Voos, W., Craig, E. & Pfanner, N. (1995). Mitochondrial GrpE modulates the function of matrix Hsp70 in translocation and maturation of proteins. *Mol. Cell. Biol.* **15**, 7098–7105.

Lew, A. M., Beck, D. J. & Thomas, L. M. (1990). Recombinant fusion proteins of protein A and protein G with glutathione S-transferase as reporter proteins. *J. Immunol. Methods,* **136**, 211–219.

Liberek, K., Marszalek, J., Ang, D. & Georgopoulos, C. (1991a). *Escherichia coli* DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. *Proc. Natl Acad. Sci. USA,* **88**, 2874–2878.

Liberek, K., Skowyra, D., Zylicz, M., Johnson, C. & Georgopoulos, C. (1991b). The *Escherichia coli* DnaK chaperone, the 70-kDa heat shock protein eukaryotic equivalent, changes conformation upon ATP hydrolysis, thus triggering its dissociation from a bound target protein. *J. Biol. Chem.* **266**, 14491–14496.

Manning-Krieg, U. C., Scherer, P. & Schatz, G. (1991). Sequential action of mitochondrial chaperones in protein import into the matrix. *EMBO J.* **10**, 3273–3280.

McCarty, J., Buchberger, A., Reinstein, J. & Bukau, B. (1995). The role of ATP in the functional cycle of the DnaK chaperone system. *J. Mol. Biol.* **249**, 126–137.

Morimoto, R. I., Tissieres, A. & Georgopoulos, C. (1994). *The Biology of Heat Shock Proteins and Molecular Chaperones . Cold Spring Harbor Monograph Series* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Nakai, M., Kato, Y., Ikeda, E., Toh-e, A. & Endo, T. (1994). YGE1p, a eukaryotic GrpE homologue, is localized in the mitochondrial matrix and interacts with mitochondrial hsp70. *Biochem. Biophys. Res. Commun.* **200**, 435–441.

Ostermann, J., Voos, W., Kang, P. J., Craig, E. A., Neupert, W. & Pfanner, N. (1990). Precursor proteins in transit through mitochondrial contact sites interact with hsp70 in the matrix. *FEBS Letters,* **277**, 281–284.

Palleros, D. R., Reid, K. L., McCarty, J. S., Walker, G. C. & Fink, A. L. (1992). DnaK, hsp73, and their molten globules. *J. Biol. Chem.* **267**, 5279–5285.

Palleros, D. R., Reid, K. L., Shi, L., Welch, W. & Fink, A. L. (1993). ATP-induced protein-hsp70 complex dissociation requires K^+ but not ATP hydrolysis. *Nature,* **365**, 664–666.

Park, H. O., Chant, J. & Herskowitz, I. (1993). *BUD2* encodes a GTPase-activating protein for Bud1/Rsr1 necessary for proper bud-site selection in yeast. *Nature,* **365**, 269–274.

Reid, K. L. & Fink, A. L. (1996). Physical interactions between members of the DnaK chaperone machinery: characterization of the DnaK.GrpE complex. *Cell Stress and Chaperones,* **1**, 127–137.

Scherer, P., Krieg, U., Hwang, S., Vestweber, D. & Schatz, G. (1990). A precursor protein partially translocated into yeast mitochondria is bound to a 70 kDa mitochondrial stress protein. *EMBO J.* **9**, 4315–4322.

Schmid, S. L., Braell, W. A. & Rothman, J. E. (1985). ATP catalyzes the sequestration of clathrin during enzymatic uncoating. *J. Biol. Chem.* **260**, 10057–10062.

Ungermann, C., Neupert, W. & Cyr, D. M. (1994). The role of Hsp70 in conferring unidirectionality on protein translocation into mitochondria. *Science,* **266**, 1250–1253.

Voos, W., Gambill, B. D., Laloraya, S., Ang, D., Craig, E. A. & Pfanner, N. (1994). Mitochondrial GrpE is present in a complex with hsp70 and preproteins in transit across membranes. *Mol. Cell. Biol.* **14**, 6627–6634..

Wang, C. & Lee, M. R. (1993). High-level expression of soluble rat hsc70 in *Escherichia coli*: purification and characterization of the cloned enzyme. *Biochem. J.* **294**, 69–77.

Wang, T.-F., Chang, J.-h. & Wang, C. (1993). Identification of the peptide binding domain of hsc70. *J. Biol. Chem.* **268**, 26049–26051.

Wei, J. & Hendershot, L. (1995). Characterization of the nucleotide binding properties and ATPase activity of recombinant hamster BiP purified from bacteria. *J. Biol. Chem.* **270**, 26670–26676.

Westerman, B., Prip-Buus, C., Neupert, W. & Schwarz, E. (1995). The role of the GrpE homologue, Mge1p, in mediating protein import and protein folding in mitochondria. *EMBO J.* **13**, 1998–2006.

Zhu, X., Zhao, X., Burkholder, W., Gragerov, A., Ogata, C., Gottesman, M. & Hendrickson, W. (1996). Structural analysis of substrate binding by the molecular chaperone DnaK. *Science,* **272**, 1606–1614.

Zylicz, M., Ang, D. & Georgopoulos, C. (1987). The GrpE protein of *Escherichia coli*. *J. Biol. Chem.* **262**, 17437–17442.

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