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ATPase Domain and Interdomain Linker Play a Key Role in Aggregation of Mitochondrial Hsp70 Chaperone Ssc1*

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Marta Blamowska[1], **Martin Sichting**[1], **Koyeli Mapa, Dejana Mokranjac, Walter Neupert, and Kai Hell**[2]

From the Adolf-Butenandt-Institut für Physiologische Chemie, Ludwig-Maximilians-Universität München, Butenandtstrasse 5, 81377 München, Germany

The co-chaperone Hep1 is required to prevent the aggregation of mitochondrial Hsp70 proteins. We have analyzed the interaction of Hep1 with mitochondrial Hsp70 (Ssc1) and the determinants in Ssc1 that make it prone to aggregation. The ATPase and peptide binding domain (PBD) of Hsp70 proteins are connected by a linker segment that mediates interdomain communication between the domains. We show here that the minimal Hep1 binding entity of Ssc1 consists of the ATPase domain and the interdomain linker. In the absence of Hep1, the ATPase domain with the interdomain linker had the tendency to aggregate, in contrast to the ATPase domain with the mutated linker segment or without linker, and in contrast to the PBD. The closest homolog of Ssc1, bacterial DnaK, and a Ssc1 chimera, in which a segment of the ATPase domain of Ssc1 was replaced by the corresponding segment from DnaK, did not aggregate in Δ*hep1* mitochondria. The propensity to aggregate appears to be a specific property of the mitochondrial Hsp70 proteins. The ATPase domain in combination with the interdomain linker is crucial for aggregation of Ssc1. In conclusion, our results suggest that interdomain communication makes Ssc1 prone to aggregation. Hep1 counteracts aggregation by binding to this aggregation-prone conformer.

Hsp70 chaperones mediate important processes in prokaryotes and eukaryotes such as folding, translocation, and prevention of aggregation of proteins (1–3). They are highly conserved in respect to both, amino acid sequence and structure. An N-terminal ATP binding domain (ATPase domain) and a C-terminal peptide binding domain (PBD)[3] are connected by a short hydrophobic interdomain linker. The PBD binds unfolded polypeptides. It acts in cooperation with the ATPase domain, which binds and hydrolyzes ATP. The nucleotide state of the ATPase domain determines the properties of the PBD. In the ATP state the PBD has a low affinity for substrates and binds and releases substrates rapidly. Hydrolysis leads to the ADP bound state of the ATPase domain in which the PBD adopts a closed conformation and binds substrates with high affinity. On the other hand, substrate binding to the PBD stimulates the hydrolysis rate of the ATPase domain. Thus, structural changes in one domain induce conformational alterations in the other domain (4–10). This interdomain communication is due to changing interdomain contacts. Such contacts have been reported for all nucleotide states (11–15); however, it is not clear whether contacts of Hsp70 proteins observed in the absence of ATP are physiologically relevant (11, 13, 15). In the ATP bound state the interdomain linker binds to a hydrophobic cleft present in the ATPase domain and stimulates the ATPase activity (15). Together with other studies this suggests an important role of the linker in the mechanism of interdomain communication (5, 15–18). Mutations within the interdomain linker inhibit the allosteric control of the ATPase domain by the PBD (5, 16, 18). DnaK constructs consisting of the ATPase domain and the interdomain linker had a higher ATPase activity than wild type DnaK and was similar to what was observed upon stimulation by substrate addition (15, 18). When the highly conserved hydrophobic residues of the linker were exchanged to alanine residues, this increase of the ATPase activity was lost (18). The interdomain linker is most likely necessary and sufficient for stimulation of the ATPase activity (15, 18).

The cycle of substrate binding and release by Hsp70 chaperones is accelerated by co-chaperones (2, 3). Members of the J co-chaperone family stimulate the ATPase activity allowing more efficient binding, whereas nucleotide exchange factors accelerate the exchange of ADP against ATP, thereby triggering release of substrates.

Mitochondria of yeast contain three members of the Hsp70 chaperone family (19–21). The most abundant one, Ssc1, mediates protein folding in the matrix space and drives translocation of proteins across the mitochondrial inner membrane. Ssq1 has a role in the biogenesis of Fe/S cluster proteins (22). For Ssc3/Ecm10, a function has not been identified (23). All mitochondrial Hsp70 chaperones work together with the nucleotide exchange factor Mge1. On the other hand, they cooperate with different J co-chaperones (19, 21, 24–26). Ssc1 uses Tim14 as J protein for protein import, and Mdj1 for protein folding processes, whereas Ssq1 employs Jac1. For being recruited to the TIM23 translocase, Ssc1, in addition, interacts with the J-related protein, Tim16, and with Tim44 in the process of protein import.

The mitochondrial Hsp70 chaperones Ssc1 and Ssq1 require the Hsp70 escort protein, Hep1 (Zim17/Tim15) (27–30). Hep1 is an L-shaped matrix protein with a zinc finger motif (31). It binds Ssc1 in mitochondria upon depletion of ATP and acts as chaperone for the Hsp70 chaperones preventing their aggrega-

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[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed. Tel.: 49-89-2180-77100; Fax: 49-89-2180-77093; E-mail: hell@med.uni-muenchen.de.

[3] The abbreviations used are: PBD, peptide binding domain; TIM, translocase of the inner membrane; aa, amino acid(s); Ni-NTA, nickel-nitrilotriacetic acid; mtHsp70, mitochondrial Hsp70.

tion (28, 29). Absence of Hep1 leads to defects in protein import by the TIM23 complex, in the morphology of mitochondria, and in biogenesis of Fe/S cluster proteins (27–30). Although a direct function of Hep1 in one of these processes cannot be excluded, all defects can be explained as secondary effects of the aggregation of mitochondrial Hsp70s that render these chaperones non-functional. The aggregation of Ssc1 and its interaction with Hep1 has been studied using purified proteins (29, 31, 32). Recombinant Ssc1 has a strong tendency to form aggregates, a process inhibited by Hep1 (29). Consistent with this observation, experiments with human Hep1 revealed binding of Hep1 to mtHsp70, with an affinity that was highest in the absence of nucleotides (32). When expressed in *E. coli*, the solubility of Ssc1/mtHsp70 and constructs of mtHsp70 were dependent on co-expression of Hep1 (29, 31, 32). Similar observations were made for chloroplast Hsp70B and its co-chaperone Hep2 (33).

It remained open, however, as to how Hep1 prevents aggregation of mitochondrial Hsp70 proteins and what makes them prone to aggregation. In particular, studies *in vivo* were lacking. To understand the mechanism of function of Hep1 we have studied its interaction with mitochondrial Hsp70 and with the various parts of mtHsp70, the ATPase domain, the PBD, and the interdomain linker. We show here that it is the ATPase domain together with the interdomain linker of Ssc1 that binds to Hep1 in mitochondria. In the absence of Hep1, the ATPase domain with the interdomain linker aggregated. We conclude that interdomain communication of mtHsp70 induces a transient aggregation-prone conformation of the ATPase domain and that Hep1 prevents mtHsp70 aggregation by binding to this conformer.

EXPERIMENTAL PROCEDURES

Plasmids—To generate expression plasmids of Ssc1 and Ssc1 derivatives, nucleotide fragments encoding full-length Ssc1 (aa 1–654), Ssc1ΔC (aa 1–633 of Ssc1 plus amino acid residues LDRV), ATPase (aa 1–411 of Ssc1), ATPase+linker (aa 1–415 of Ssc1), and ATPase+linkerA4 (aa 1–411 of Ssc1 plus amino acid residues AAAA) with C-terminal His_6 tags were amplified by polymerase chain reaction. Additionally fragments encoding versions of Ssc1ΔC and ATPase+linker without the His tag were amplified. All DNA fragments were cloned into pYX142 vector using EcoRI and XhoI restriction sites. The plasmid pRS315-Ssc1PromPresDnaK was generated in two steps. First, the nucleotide sequence for the promoter and the presequence encoding region of SSC1 (nucleotides −497 to 69) was amplified by PCR using yeast genomic DNA as template and the obtained fragment was cloned into vector pRS315 using SacI and BamHI restriction sites. Then the amplified coding sequence of DnaK plus a C-terminal His_6 tag was cloned as BamHI/XhoI fragment into the vector. The plasmid for Ssc3 overexpression was obtained by replacing the nucleotide sequence encoding DnaK with the sequence encoding mature Ssc3 (amino acid residues 21 to 644). To obtain the $Ssc1K_{ins}$ mutant, nucleotide sequences of the *SSC1* gene (−497–705 and 730–1962) were amplified using the primer pairs 5′-SacI-Ssc1Prom/3′-NheI-Kins and 5′-NheI-Kins/3′-XhoI-Ssc1his. The primers 3′-NheI-Kins and 5′-NheI-Kins contained nucleotide sequences encoding amino acid residues 209–220 of DnaK. The fragments were digested with the indicated restriction enzymes and cloned with SacI and XhoI into the vector pRS315. The plasmid pYX142-PBD-His was generated by cloning a fragment containing the coding sequences for the presequence of subunit 9 of the F_1F_O-ATPase of *Neurospora crassa* and for amino acid residues 410–654 of Ssc1 via restriction sites EcoRI and XhoI into vector pYX142. The fragment was obtained by PCR using plasmid pYX132-PBD as template (34). The plasmids pYX132-A1P3 and pYX132-A3P1 contained the nucleotide sequences encoding full-length Hsp70 proteins consisting of either the ATPase domain of Ssc1 and the PBD of Ssc3 (A1P3) or vice versa (A3P1).

Strains and Cell Culture—The haploid Δ*hep1* deletion yeast strain and the corresponding wild type FY1679-11a were obtained from the Euroscarf strain collection (accession numbers 10179B and 10000R, respectively) and used to obtain the derivatives expressing DnaK, Ssc3, and Ssc1 variants. The strains *ssc1–2*, *ssc1–3*, and *ssc1ΔC*, which harbor Ssc1 lacking the last 21 amino acid residues were as described (35, 36). For plasmid shuffling the Δ*ssc1* deletion strain harboring a *URA3* containing plasmid expressing a C-terminal His-tagged form of Ssc1 was used (37). Yeast cells were grown at 24 or 30 °C on lactate medium or selective medium containing 2% glucose (38).

Expression and Coexpression in E. coli—The pETDuet-1 system (Novagen) was used for expression of DnaK, Ssc1 (29), Ssc3/Ecm10, $SscK_{ins}$, and the ATPase domain, ATPase+linker, ATPase+linkerA4, and PBD of Ssc1 in *E. coli*. To express the proteins, nucleotide sequences encoding DnaK, as well as mature forms of Ssc3, $SscK_{ins}$, and the Ssc1 constructs were cloned as NdeI/XhoI fragments into the pETDuet-1 vector generating pETDuet-DnaK, pETDuet-Ssc3, pETDuet-SscKins, pETDuet-PBD, pETDuet-ATPase, pETDuet-ATPase+linker, and pETDuet-ATPase+linkerA4. For co-expression of Hep1, the nucleotide sequence coding for the mature form of Hep1 (amino acid residues 74–205) was cloned as a BamHI/HindIII fragment into pETDuet-1 plasmids containing sequences coding for the Ssc3 and Ssc1 constructs. In these constructs, the Hep1 protein contained an N-terminal His_6 tag. Plasmids were transformed into *Escherichia coli* BL21(DE3) cells. Expressed proteins were analyzed for solubility as previously reported (29).

Aggregation Assay—Mitochondria isolated from yeast cells were preincubated at 25 °C for 15 min and then solubilized with buffer containing 1% (w/v) Triton X-100, 20 mM Tris, pH 7.4, 80 mM KCl, and 1 mM phenylmethylsulfonyl fluoride for 30 min on ice. Half of the sample was removed as control of total protein. The other half was centrifuged at 16,000 × *g* for 10 min at 4 °C to separate soluble (supernatant) and insoluble (pellet) fractions. Total and supernatant were precipitated with trichloroacetic acid. Samples were analyzed by SDS-PAGE and immunodecoration.

Chemical Cross-linking—Chemical cross-linking was performed essentially as described by Mokranjac *et al.* (39). Mitochondria were incubated for 10 min at 37 °C in import buffer with addition of 10 units/ml of apyrase and 10 μM oligomycin to deplete the ATP. The cross-linker disuccinimidyl glutarate was

added to a final concentration of 150 μM. Samples were incubated for 30 min on ice and then quenched by addition of 0.1 M glycine, pH 8.8. Mitochondria were reisolated and cross-linked adducts were analyzed by SDS-PAGE and immunodecoration.

Ni-NTA-agarose Pull-down Assay and Co-immunoprecipitation—For Ni-NTA-agarose pull-down mitochondria were depleted of ATP (see above) and solubilized at 1 mg of protein/ml with 1% (w/v) Triton X-100 in buffer A (20 mM Tris, 80 mM potassium acetate, 20 mM imidazole, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, pH 8.0) for 30 min at 4 °C. After a clarifying spin, solubilized material was incubated with 50 μl of Ni-NTA-agarose beads for 1 h. Following removal of the supernatant, beads were washed 3 times with buffer A containing 0.05% Triton X-100 and bound proteins were eluted with Laemmli buffer containing 300 mM imidazole. Total, supernatant, and elution fractions were then subjected to SDS-PAGE and immunodecoration. For co-immunoprecipitation experiments, mitochondria were subjected to ATP depletion followed by solubilization in buffer containing 20 mM Tris, pH 7.4, 80 mM potassium acetate, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1% digitonin for 30 min on ice. Mitochondrial lysates were further processed as described by Mokranjac *et al.* (40).

RESULTS

All Mitochondrial Hsp70 Family Members Have the Propensity to Aggregate—Mitochondrial Hsp70 proteins Ssc1 and Ssq1 have the tendency to aggregate in the absence of their co-chaperone Hep1 (28, 29). To address whether all mitochondrial Hsp70 proteins are prone to aggregation we studied the third member of the Hsp70 family of yeast mitochondria, Ssc3/Ecm10. Because the endogenous levels of Ssc3 are very low, it was expressed with a C-terminal His_6 tag under control of the Ssc1 promotor to analyze its solubility. About 40% of Ssc3 were found in the insoluble fraction (Fig. 1*A*). Thus, Ssc3, in contrast to Ssc1 and Ssq1, aggregates in mitochondria in the presence of Hep1. This was not due to limiting amounts of Hep1 because of overexpression of Ssc3, because Ssc1 was still soluble in these mitochondria. We analyzed the interaction of Ssc3 with Hep1 under conditions in which Ssc1 binds to Hep1. Mitochondria of the strain expressing His-tagged Ssc3 were depleted of ATP, solubilized with Triton X-100, and incubated with Ni-NTA beads. His-tagged Ssc3 and the interaction partner Mge1, but not Hep1 were bound to the Ni-NTA beads (Fig. 1*B*). Apparently, Hep1 does not interact with Ssc3. Consistent with these results, Ssc3 did not become soluble in *E. coli* cells upon co-expression of Hep1, whereas Ssc1 did, as previously reported (Fig. 1*C*) (29). In conclusion, all mitochondrial Hsp70 proteins are prone to aggregation. Ssc3, in contrast to Ssc1 and Ssq1, partly aggregates in the presence of Hep1, probably due to its inability to associate with Hep1.

The ATPase Domain of Ssc1 Is Essential for Its Interaction with Hep1—The inability of Ssc3 to associate with Hep1 was used to identify the domain of Ssc1 interacting with Hep1. Chimeric proteins containing the ATPase domain of Ssc1 and the PBD of Ssc3 (A1P3) or the ATPase domain of Ssc3 and the PBD of Ssc1 (A3P1) were constructed and expressed in wild type yeast cells. The chimeric proteins were immunoprecipitated

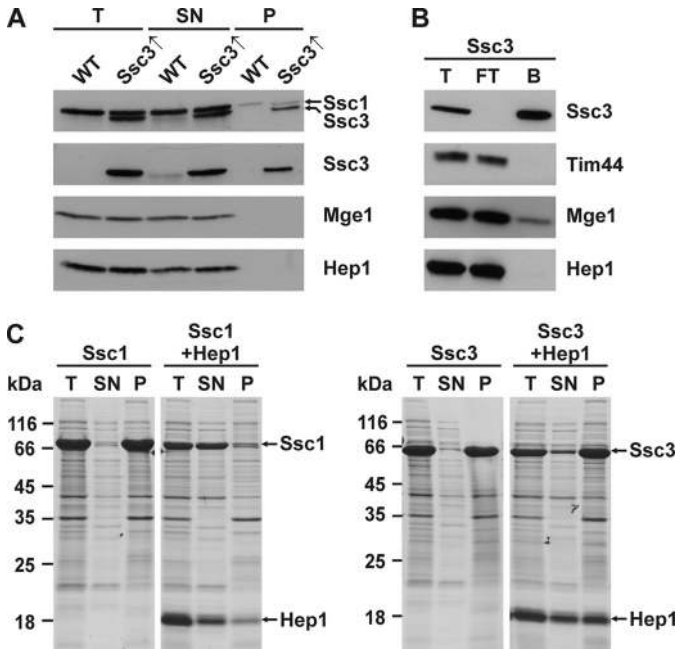

FIGURE 1. **Ssc3 aggregates upon overexpression in mitochondria.** *A*, mitochondria isolated from a wild type yeast strain and a strain overexpressing His-tagged Ssc3 were solubilized with Triton X-100. Soluble proteins (*SN*) and aggregated fractions (*P*) were separated by centrifugation. These fractions and total material (*T*) were analyzed by SDS-PAGE and immunodecoration with antibodies against the indicated proteins. *B*, mitochondria from a strain overexpressing His-tagged Ssc3 were depleted of ATP and solubilized with Triton X-100. Mitochondrial lysate was incubated with Ni-NTA beads. Bound proteins were eluted with Laemmli buffer containing 300 mM imidazole. Samples were analyzed by SDS-PAGE and immunodecoration with antibodies against the indicated proteins. Fractions of total (*T*) and unbound (*FT*) proteins represent 20% of the material present in the bound fraction (*B*). *C*, Ssc1 and Ssc3 were either expressed alone or co-expressed with Hep1 in *E. coli* cells grown at 37 °C. Cells were lysed and one-half was removed as total proteins (*T*). The other half was separated by centrifugation into soluble (*SN*) and insoluble (*P*) proteins. Samples were analyzed by SDS-PAGE and Coomassie staining.

from lysates of mitochondria depleted of ATP. Hep1 could be detected only in the precipitate from extracts containing A1P3, whereas Mge1 was co-precipitated with both chimeric proteins (Fig. 2*A*). These results suggest an interaction of Hep1 with the ATPase domain of Ssc1. Further experimental evidence came from the analysis of Ssc1 mutants, *ssc1–2* and *ssc1–3* (35, 36). *Ssc1–2* carries a single point mutation in the PBD, whereas *ssc1–3* has a single mutation in the ATPase domain. Similar amounts of Hep1, Tim44, and Mge1 were detected in the immunoprecipitate from extracts of wild type and *Ssc1–2* mitochondria. In contrast, hardly any Hep1 was co-precipitated from *Ssc1–3* mitochondrial extracts (Fig. 2*B*). Hep1 behaved like Mge1, a protein known to bind to the ATPase domain. Thus, the interaction of Ssc1 with Hep1 depends on the ATPase domain of Ssc1.

The ATPase Domain with Hydrophobic Linker but Not the ATPase Domain Binds Hep1—Is the ATPase domain of Ssc1 sufficient for interaction with Hep1? We tested the individual domains of Ssc1 for binding to Hep1. To this end, the ATPase domain, the PBD with the interdomain linker at its N terminus, referred to as PBD, were expressed as His-tagged variants in wild type cells and tested for interaction with Hep1. Following ATP depletion mitochondria were lysed and the mitochondrial

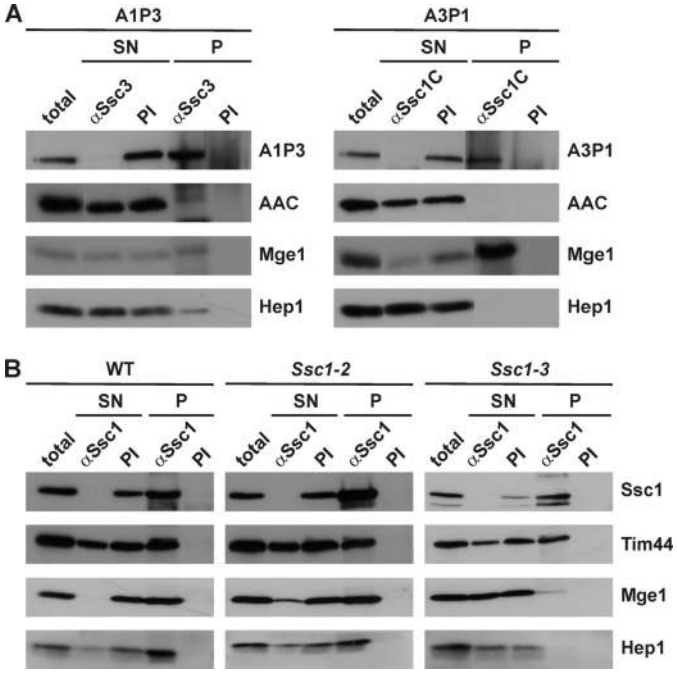

FIGURE 2. **The ATPase domain of Ssc1 is required for interaction with Hep1.** *A*, mitochondria isolated from *ssc1ΔC* strains expressing the A1P3 chimera (*left panel*) or the A3P1 chimera (*right panel*) were depleted of ATP and solubilized with Triton X-100. Mitochondrial lysates were subjected to co-immunoprecipitation with the antibodies against Ssc3 and Ssc1 or preimmune IgGs (*PI*) as control. Bound material (*P*) was eluted with Laemmli buffer and the samples were analyzed by SDS-PAGE and immunodecoration with antibodies specific for the indicated proteins. The antibodies directed against the C-terminal peptide of Ssc1 (*αSsc1C*) allowed specific precipitation and detection of the chimera A3P1. *SN*, unbound proteins. *B*, mitochondria were isolated from a wild type yeast strain and temperature-sensitive mutant strains *ssc1–2* and *ssc1–3* grown at a permissive temperature of 24 °C. Following ATP depletion, mitochondria were solubilized with Triton X-100 and subjected to coimmunoprecipitation with antibodies directed against Ssc1 or preimmune IgGs (*PI*) as control. The samples were analyzed as described in *A*.

extracts were incubated with Ni-NTA beads and bound proteins were analyzed by SDS-PAGE and immunodecoration with antibodies against Ssc1 and Hep1. Hep1 was co-purified with full-length Ssc1 as a control, but neither with the ATPase domain nor with the PBD (Fig. 3*A*, *upper panel*). Although the ATPase domain is required for interaction with Hep1, it appears not to be sufficient. This was supported by cross-linking experiments with purified components. Whereas a cross-linked adduct between Hep1 and full-length Ssc1 was observed, no adduct between the ATPase domain and Hep1 was detected (data not shown). We conclude that Hep1 recognizes the ATPase domain of Ssc1 when it is present in the context of additional parts of the protein.

Because the interdomain linker is known to influence properties of the ATPase domain in DnaK (15, 18), we asked whether a Ssc1 construct consisting of the ATPase domain with linker (ATPase+linker) binds Hep1. As control, we tested a construct (ATPase+linkerA4) in which 4 amino acid residues of the interdomain linker (VLLL) were replaced by alanine residues. Such a linker mutant did not affect the activity of the ATPase domain in the case of DnaK (18). We purified both constructs via their C-terminal His tags from mitochondrial extracts depleted of ATP. Whereas Hep1 was not associated with ATPase+linkerA4, it was associated with the isolated ATPase+linker construct, although with reduced efficiency compared with its association with full-length Ssc1 (Fig. 3*A*, *lower panel*). This interaction was specific, because Hep1 was not found in the eluate fraction of extracts containing the Ssc1 variant lacking a His tag. Similar to full-length Ssc1, the ATPase domain with the linker could be cross-linked to Hep1 supporting the co-isolation experiments (Fig. 3*B*). A cross-linked adduct was neither observed for the ATPase domain, nor for the ATPase+linkerA4 construct. The isolation of the cross-linked adducts with Ni-NTA beads confirmed that Hep1 was cross-linked to the His-tagged Ssc1 and the His-tagged ATPase+linker construct (Fig. 3*C*, *lanes 4* and *12*, respectively). No adducts containing Hep1 were observed in bound fractions upon isolation of the His-tagged constructs of the ATPase domain and linker mutant ATPase+linkerA4. We conclude that the ATPase domain with the interdomain linker is necessary and sufficient for binding of Hep1.

The Solubility of the ATPase Domain with Linker Is Dependent on Hep1—What are the structural properties of Ssc1 that make its solubility in mitochondria dependent on Hep1? The importance of the ATPase domain for interaction with Hep1 suggests a role of Hep1 in protection of the ATPase domain against aggregation. When we expressed the ATPase domain and the PBD in wild type and in a strain lacking Hep1, both domains were found in the soluble fractions even in the absence of Hep1 (Fig. 4*A* and data not shown). On the contrary, the Ssc1 protein and a functional Ssc1 variant lacking the C-terminal 21 amino acid residues aggregated in the absence of Hep1. Thus, neither the ATPase domain nor the PBD have the propensity to aggregate. It appears that domain communication within the Ssc1 protein triggers conformational changes leading to aggregation. Interactions that mediate domain communication have been reported for the short hydrophobic interdomain linker and the ATPase domain (5, 15–18). The construct consisting of the ATPase domain and the linker binds to Hep1 suggesting an effect of Hep1 on this construct. Indeed, the construct was found soluble in wild type mitochondria, whereas a fraction of this construct was found in the pellet fraction, when expressed in cells lacking Hep1 (Fig. 4*B*). The linker sequence clearly had influence on the solubility of the construct, because the ATPase+linkerA4 construct harboring a mutated linker segment did not aggregate in mitochondria isolated from wild type and Δ*hep1* cells. This suggests that the ATPase domain in combination with the linker adopts an aggregation-prone conformation and that Hep1 is capable of preventing this aggregation.

The dependence of the solubility of Ssc1 on Hep1 was analyzed in *E. coli*. As reported previously, aggregation of full-length Ssc1 in *E. coli* cells was counteracted by co-expression of Hep1 (Fig. 4*C*, *upper left panel*) (29, 31, 32). Here we used this assay system to test the solubility of the ATPase domain, the PBD, the ATPase+linker, and the ATPase+linkerA4 linker variant in the absence and presence of Hep1 (Fig. 4*C*). The PBD was soluble even without co-expression of Hep1 indicating the correct folding of this domain independently of Hep1 (Fig. 4*C*, *upper middle panel*). In contrast, the ATPase domain was found in the pellet fraction independent of the presence of Hep1 (Fig. 4*C*, *upper right panel*). Aggregation was observed when the ATPase domain plus interdomain linker was

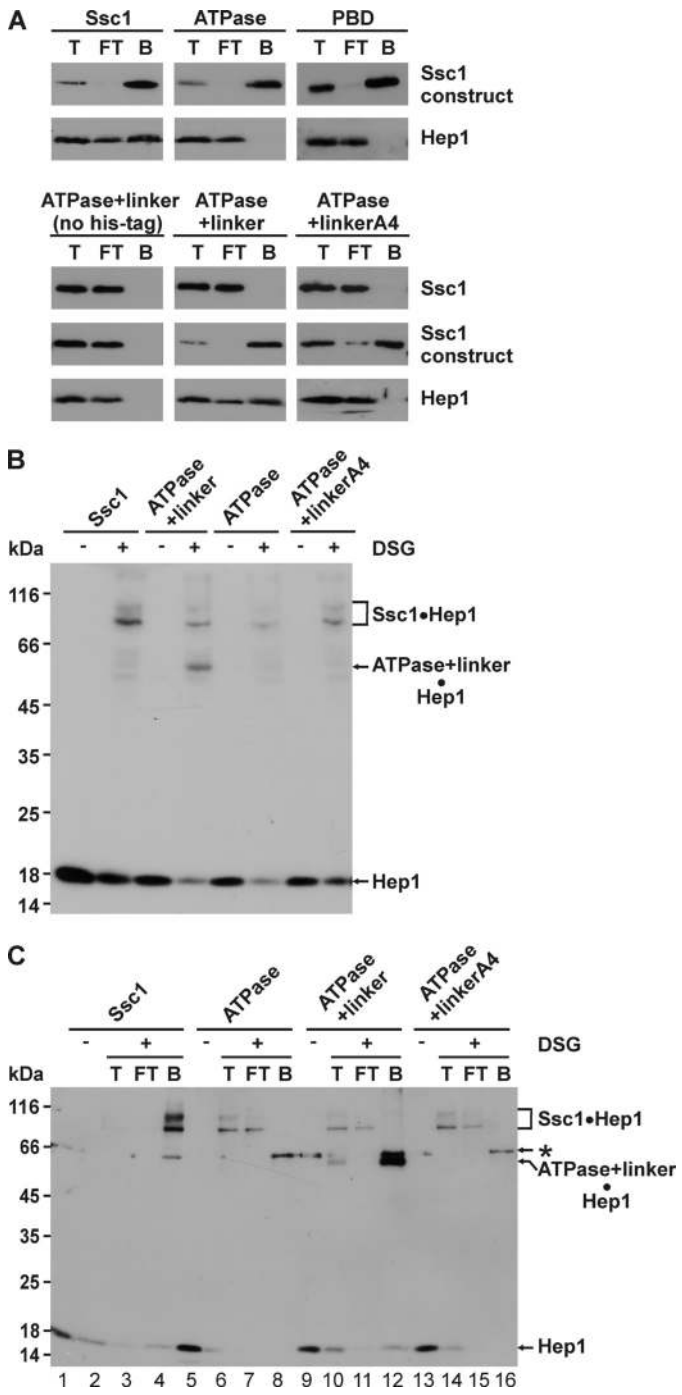

FIGURE 3. **The ATPase domain together with the interdomain linker, but not the ATPase domain alone of Ssc1 interacts with Hep1.** *A*, mitochondria from strains expressing a His-tagged construct of either full-length Ssc1 or the ATPase domain or the PBD of Ssc1 (*upper panel*) were depleted of ATP. In addition, ATP was depleted in mitochondria from strains expressing a construct of Ssc1 containing the ATPase domain and interdomain linker without or with a His tag (ATPase+linker (no His tag) or ATPase+linker, respectively) or a His-tagged construct of the ATPase domain with a mutated linker segment, in which amino acid residues VLLL were replaced by four alanine residues (ATPase+linkerA4) (*lower panel*). After solubilization with Triton X-100, Ni-NTA pull-down was carried out as described in Fig. 1*B*. *T*, total proteins (20%); *FT*, unbound material (20%); and *B*, bound proteins (100%) were analyzed by SDS-PAGE and immunodecoration with antibodies specific for the indicated proteins. *B*, mitochondria were isolated from strains expressing the indicated constructs of Ssc1. ATP-depleted mitochondria were incubated with or without the chemical cross-linker disuccinimidyl glutarate (*DSG*). Cross-linked adducts of Hep1 were analyzed by SDS-PAGE and immunodecoration with antibodies against Hep1. *C*, mitochondria were subjected to cross-linking as in *B*, but then not directly analyzed by SDS-PAGE but solubilized with Triton X-100 and incubated with Ni-NTA beads. Bound protein adducts were eluted with Laemmli buffer containing 300 mM imidazole. Non-treated control samples (−*DSG*, 5%), and cross-linker-treated samples (total (*T*, 5%), unbound material (*FT*, 5%), and bound proteins (*B*, 100%)) were analyzed by SDS-PAGE and immunodecoration with antibodies against Hep1. The adduct of Ssc1 with Hep1 and the adduct of the ATPase+linker and Hep1 are indicated; the *asterisk* indicates cross-reaction of the antibodies.

expressed (Fig. 4*C*, *lower left panel*). However, upon co-expression of Hep1 most of the ATPase domain plus linker was recovered in the soluble fraction. In contrast, the ATPase+linkerA4 variant was not recovered in the soluble fraction upon co-expression of Hep1 (Fig. 4*C*, *lower right panel*). We conclude that Hep1 prevents aggregation of the ATPase plus interdomain linker variant of Ssc1 consistent with the observations made in yeast.

The Exchange of a Short Segment of Amino Acid Residues in the ATPase Domain of Ssc1 Generates a Soluble Non-aggregating Protein—Do all members of the Hsp70 family require Hep1 to prevent their aggregation in mitochondria? If this is the case, non-mitochondrial homologs of Ssc1 are expected to aggregate when targeted to Δ*hep1* mitochondria. To test this we expressed the closest non-mitochondrial homolog, DnaK of *E. coli*, with the mitochondrial matrix targeting signal of Ssc1 and a C-terminal His_6 tag under control of the Ssc1 promotor in wild type and Δ*hep1* cells. DnaK was found in the soluble fraction of mitochondria isolated from both strains (Fig. 5*A*). DnaK did not aggregate in mitochondria in the absence of Hep1. Thus, Hep1 is not required to prevent aggregation of every Hsp70 protein sorted to mitochondria. The result showed that post-translational import of Hsp70 proteins into mitochondria does not cause their aggregation in the absence of Hep1. The propensity to aggregate appears to be a specific feature of the mitochondrial Hsp70 proteins rather than a common feature of all Hsp70 proteins.

To identify parts in the ATPase domains of mitochondrial Hsp70s that contribute to their aggregation we compared the amino acid sequences of mitochondrial Hsp70s from different species with the amino acid sequence of DnaK from *E. coli* (Fig. 5*B*). Although these sequences are highly conserved, there are some differences, *e.g.* an insertion of four amino acid residues in DnaK (residues 212–215). This insertion is located in a region of the ATPase domain that was proposed to be involved in interdomain communication with the PBD of Hsp70s (12). Also residues N-terminal (residues 209 and 211 of DnaK) and C-terminal (amino acid residues 219 and 220) to the insertion differ between mtHsp70s and DnaKs.

We constructed a mutant $SscK_{ins}$, a variant of Ssc1 that has residues 236–243 of Ssc1 replaced by residues 209–220 of DnaK. Cells expressing $SscK_{ins}$ instead of Ssc1 were able to grow on fermentable carbon sources at low temperatures, but not at 30 °C and above (data not shown). Thus, the $SscK_{ins}$ variant is present in a functional conformation and can partially complement the deletion of *SSC1*.

To study solubility of the $SscK_{ins}$ variant, it was expressed in wild type and Δ*hep1* cells. In contrast to Ssc1ΔC, $SscK_{ins}$ was recovered in the soluble fraction of mitochondria from both cells (Fig. 5*C*, data not shown). These data indicate that the

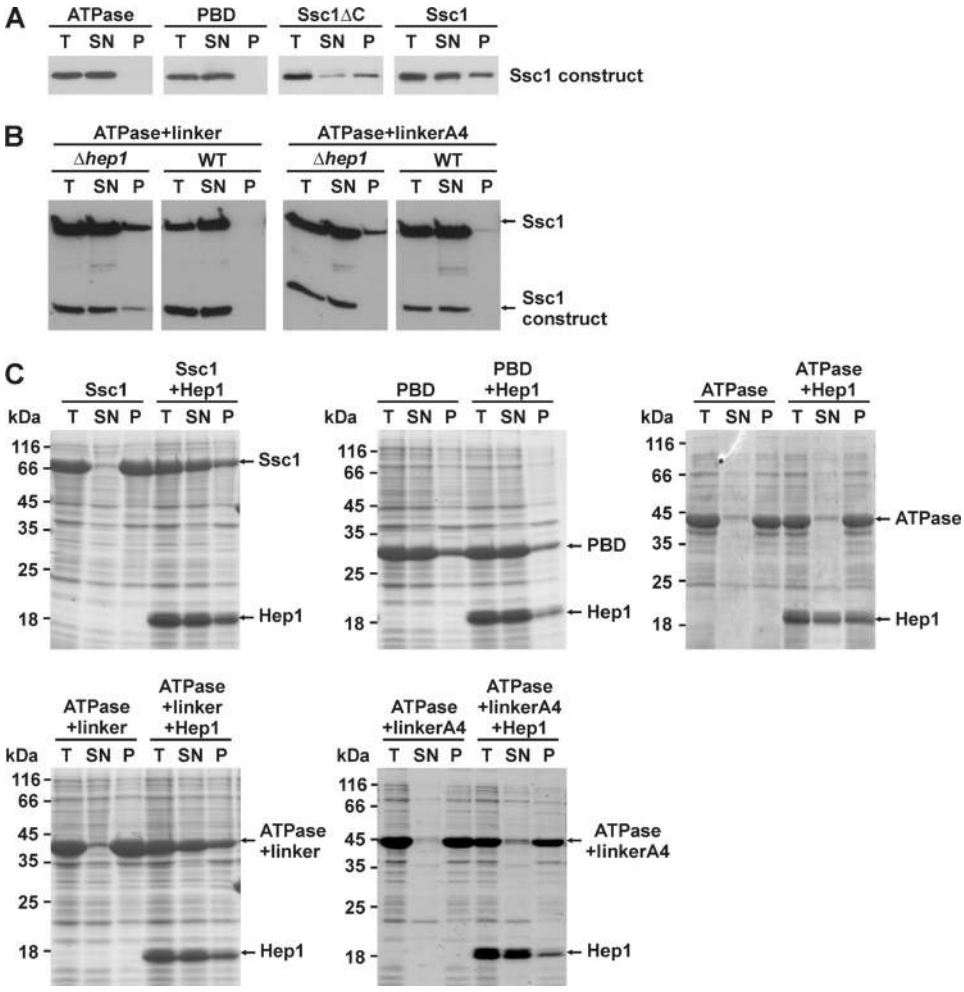

FIGURE 4. **In contrast to the ATPase domain and PBD, the ATPase+linker protein aggregates in the absence of Hep1.** *A*, mitochondria from Δ*hep1* strains expressing His-tagged versions of ATPase domain, PBD, Ssc1ΔC, or full-length Ssc1 were solubilized with Triton X-100. Soluble (*S*) and insoluble (*P*) fractions separated by centrifugation, as well as total material (*T*) were subjected to SDS-PAGE and immunodecoration. *B*, mitochondria were isolated from Δ*hep1* and wild type strains expressing His-tagged versions of the ATPase+linker and as control the ATPase+linkerA4 construct of Ssc1, grown at 30 °C. Aggregation was assayed as described in *A*. *C*, the indicated constructs of Ssc1 were either expressed alone or coexpressed with Hep1 in *E. coli* cells grown at 37 °C. Cells were lysed and one-half was removed as total proteins (*T*). The other half was separated by centrifugation into soluble (*SN*) and insoluble (*P*) proteins. Samples were analyzed by SDS-PAGE and Coomassie staining.

exchange of amino acid residues 209–220 of DnaK with the respective segment of Ssc1 increases the solubility of the chimera in the absence of Hep1.

Because $SscK_{ins}$ did not aggregate in the absence of Hep1, we expected it not to interact at all with Hep1. Indeed, $SscK_{ins}$ interacted with Tim44 and Mge1 in amounts comparable with Ssc1, but virtually no Hep1 was bound by $SscK_{ins}$ (Fig. 5*D*). Thus, the insertion of the DnaK segment did not only increase the solubility of the $SscK_{ins}$ protein, but also strongly affected the interaction with Hep1.

These results are supported by data obtained in *E. coli* cells. $SscK_{ins}$, Ssc1, or DnaK were expressed in *E. coli* cells and their solubilities tested. In contrast to Ssc1, DnaK and $SscK_{ins}$ were recovered in the soluble fraction (Fig. 5*E*). Thus, $SscK_{ins}$ did not aggregate. These observations imply that insertion of residues 209 to 220 of DnaK into Ssc1 increase its solubility in the absence of Hep1. In summary, this segment in the ATPase domain affects the propensity of Ssc1 to aggregate. Our findings suggest that intramolecular interactions mediated by this segment of the ATPase domain trigger aggregation of Ssc1.

DISCUSSION

In this study we have analyzed the molecular determinants responsible for the propensity of mitochondrial Hsp70 to aggregate and for binding of mitochondrial Hsp70 to its escorting factor Hep1. Our results show that the ATPase domain is the domain of mitochondrial Hsp70s that is crucial for aggregation. This domain alone, however, did not aggregate in yeast in the absence of Hep1. In contrast, the ATPase domain with the adjacent interdomain linker was prone to aggregation. The addition of the linker segment to the ATPase domain not only led to its aggregation, but also to binding to Hep1. Nevertheless, it is unlikely that simply the linker mediates interaction with Hep1 and aggregation in the absence of Hep1. When the linker segment was present in front of the PBD, the protein was soluble and did not interact with Hep1. In addition, DnaK neither interacts with Hep1 nor aggregates, although the sequence of the interdomain linker is highly conserved between mitochondrial Hsp70s and bacterial DnaK. The same is true for the $SscK_{ins}$ variant, which contains the interdomain linker of Ssc1.

What is then responsible for the aggregation of Ssc1? Interdomain communication between the ATPase domain and the PBD of members of the Hsp70 family is well documented (4–10). The interdomain linker plays an important role in this communication and is sufficient to exert effects on the ATPase domain, such as stimulation of its ATPase activity. Thus, our results suggest that interaction of the interdomain linker with the ATPase domain induces an aggregation-prone conformation of the protein.

As shown in the model in Fig. 6, the ATPase domain of Ssc1 goes through an aggregation-prone conformation. This conformation might be formed during *de novo* folding and/or during the reaction cycle of Ssc1. Hep1 binds to this conformer, thereby preventing aggregation of Ssc1. In the absence of Hep1, Ssc1 molecules present in this conformation accumulate in aggregates. The conformation of Ssc1 in the aggregates may differ from the aggregation-prone conformation. Future studies will have to elucidate the exact nature of the interaction between Ssc1 and Hep1.

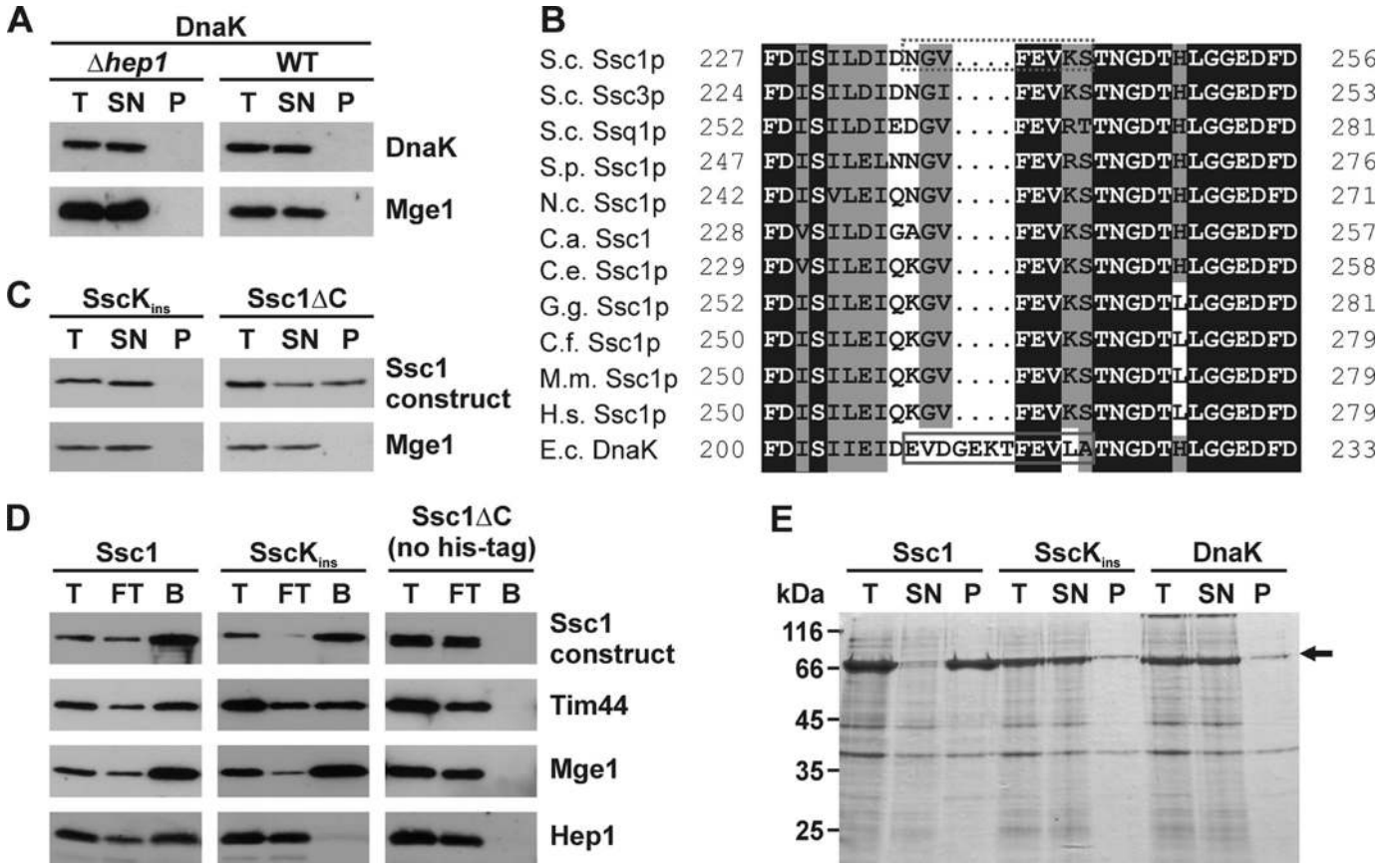

FIGURE 5. **Replacement of amino acid residues 236–243 of Ssc1 by amino acid residues 209–220 of DnaK leads to increased solubility of the resulting $SscK_{ins}$ variant.** *A*, mitochondria were isolated from Δ*hep1* and wild type yeast strains expressing His-tagged DnaK. Aggregation of proteins was tested as described in Fig. 1*A* and samples were analyzed by SDS-PAGE and immunodecoration with antibodies against DnaK and Mge1. *B*, alignment of segments from the ATPase domains of mitochondrial Hsp70s and *E. coli* DnaK. The positions of the first and last amino acid residue of these segments are indicated. The alignment was generated using the software DNAMAN 4.22. Identical residues are shown in *black*, similar residues in *gray*. *Dotted* and *solid frames* indicate the residues of Ssc1 removed and the residues of DnaK inserted in the $SscK_{ins}$ construct, respectively. *S.c.*, *Saccharomyces cerevisiae*; *S.p.*, *Schizosaccharomyces pombe*; *N.c.*, *Neurospora crassa*; *C.a.*, *Candida albicans*; *C.e.*, *Caenorhabditis elegans*; *G.g.*, *Gallus gallus*; *C.f.*, *Canis lupus familiaris*; *M.m.*, *Mus musculus*; *H.s.*, *Homo sapiens*; *E.c.*, *Escherichia coli*. *C*, the His-tagged variants $SscK_{ins}$ and Ssc1ΔC as control were expressed in the Δ*hep1* strain and tested for aggregation as described in the legend to Fig. 1*A*. For detection of Ssc1 constructs antibodies directed against the His tag were used. *D*, mitochondria isolated from Δ*ssc1* strains expressing His-tagged Ssc1 or His-tagged $SscK_{ins}$, as well as mitochondria isolated from the wild type strain expressing non-tagged Ssc1ΔC as control were depleted of ATP and the Ni-NTA pull-down assay was performed as described in the legend to Fig. 1*B*. Total (*T*) and unbound proteins (*FT*) represent 20% of bound proteins (*B*). Samples were analyzed by SDS-PAGE and immunodecoration with antibodies against the indicated proteins. *E*, Ssc1, $SscK_{ins}$, and DnaK were expressed in *E. coli* cells grown at 24 °C. Cells were lysed and one-half was removed as total proteins (*T*). The other half was separated by centrifugation into soluble (*SN*) and insoluble (*P*) proteins. Samples were analyzed by SDS-PAGE and Coomassie staining. The *arrow* indicates the expressed proteins.

Based on the data obtained with the Ssc1 variant containing the extended segment of DnaK ($Ssc1K_{ins}$) a prediction on the region in the ATPase domain contributing to the propensity of aggregation of Ssc1 is suggested. The corresponding region in bovine Hsc70 has been shown to contribute to the interface of the ATPase with the PBD (12). Because amino acid residues of the domain interfaces are highly conserved between bovine Hsc70 and Ssc1, similar contacts are likely for Ssc1. As reported here, replacement of this segment by the corresponding segment of DnaK obviously changes the properties of Ssc1 and makes it less prone to aggregation. An invariant positively charged amino acid residue, in Ssc1 lysine at position 242, was exchanged to a leucine residue and an additional stretch of four residues (GEKT) was introduced by the replacement in this study. The insertion of the GEKT stretch expands the loop in front of the interface β-sheet of the ATPase domain (residues 238–248) based on the modeled structure of $SscK_{ins}$, which was obtained using the bovine Hsc70 crystal structure and the SWISS-MODEL program (41). Thereby this loop draws nearer to the interdomain linker between the ATPase domain and PBD. Thus, it might allow stabilization of the interdomain linker leading to a conformation that has a reduced tendency to aggregate. The exchange of Lys to Leu might support hydrophobic interactions. Mutant variants within this segment will be helpful in the future to determine the contribution of individual structural elements in this segment to the solubility of Ssc1. The importance of this region for Ssc1 function has been confirmed by observations made with an E240A/V241A mutant. The mutant protein was not functional in intact yeast cells (42). This segment in the ATPase domain, however, is probably not the only region crucial for aggregation of Ssc1, because the $Ssc1K_{ins}$ variant was observed to aggregate in *E. coli* at higher temperatures (data not shown).

The ATPase domain with linker, when expressed in *E. coli*, was found to be soluble in an Hep1-dependent manner, whereas the ATPase domain alone was insoluble in the absence and presence of Hep1. Apparently, solubility of the ATPase domain differs in yeast and *E. coli* suggesting differences

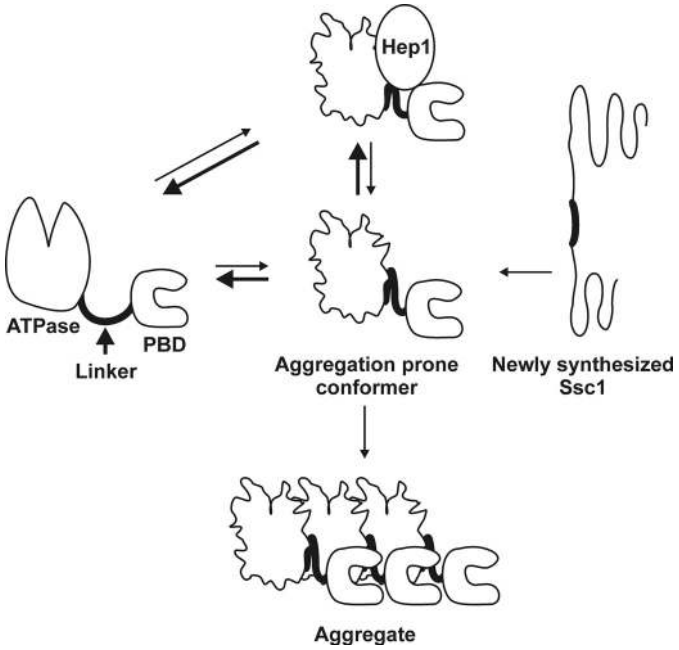

FIGURE 6. **Model of the action of Hep1 to prevent aggregation of Ssc1.** Ssc1 can adopt an aggregation-prone conformation. This conformation may be formed during *de novo* folding of newly synthesized Ssc1 and/or from folded Ssc1. Interdomain communication between the PBD and the ATPase domain is prerequisite for reaching this conformation. The interdomain linker, which is important for communication between both domains, is sufficient to induce the aggregation-prone conformation of the ATPase domain. Hep1 binds to this conformer. Release of Hep1 most likely occurs after Ssc1 has been folded to reach a state not any longer prone to aggregation. It is also possible that refolding to its native form takes place via the aggregation-prone conformation following release of Hep1. In the absence of Hep1, aggregation-prone Ssc1 accumulates and aggregates to large assemblies.

between the processes of aggregation in yeast and bacteria. This might explain why nucleotide exchange factor Mge1 cannot keep mtHsp70 soluble in yeast in the absence of Hep1, although its co-expression in *E. coli* renders mtHsp70 soluble (31). Thus, the *E. coli* cell is a valuable test system for Hep1 dependent solubility of mtHsp70, but investigations under physiological conditions in yeast are still necessary. Aggregation of human mtHsp70 in *E. coli* was also observed, but in contrast to the results presented here Hep1 was reported to bind to and render the ATPase domain of mtHsp70 soluble (32). However, in fact an ATPase domain with linker was used.

Hep proteins are required in mitochondria, organelles evolutionary derived from bacteria. They appear to have evolved to keep Hsp70 proteins soluble that have, in contrast to their bacterial counterparts, the propensity to aggregate. Why have the mitochondrial Hsp70 proteins developed a tendency to aggregation? They might have done so to be able to exert additional functions acquired during the evolution of mitochondria. DnaK was reported not to be able to rescue the deletion of Ssc1 (34), although it is soluble in mitochondria lacking Hep1. Functional interactions of DnaK with the mitochondrial co-chaperones needed for protein folding, Mdj1 and Mge1, have been reported *in vitro* and *in vivo* (43–45). Previous reports showed, however, that DnaK does not interact with the translocase component Tim44 in the same manner as Ssc1 and does not promote import of preproteins into mitochondria, a process that must have evolved during endosymbiosis of a bacterial ancestor (34). The function in the import motor and/or other processes might depend on a more flexible conformation of the mtHsp70 protein that on the other hand would lead to the propensity for self-aggregation.

Homologs of yeast Hep1 have been identified in the genomes of higher eukaryotes. As already stated, human Hep1 has been shown to keep mtHsp70 soluble and functional (32). In addition, human Hep1 may have a stimulatory effect on the catalytic activity of the ATPase domain of mtHsp70. Interestingly, a Hep2 homolog has been identified in chloroplasts in the algae *Chlamydomonas reinhardtii* (33). This raises intriguing questions on the evolutionary origin of Hep proteins and their functions in the two organelles.

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