Mitochondrial Hsp70 Ssc1: Role in Protein Folding*

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Ssc1, the major Hsp70 of the mitochondrial matrix, is involved in the translocation of proteins from the cytosol into the matrix and their subsequent folding. To better understand the physiological mechanism of action of this Hsp70, we have undertaken a biochemical analysis of Ssc1 and two mutant proteins, Ssc1–2 and Ssc1–201. ssc1–2 is a temperature-sensitive mutant defective in both translocation and folding; ssc1–201 contains a second mutation in this ssc1 gene that suppresses the temperature-sensitive growth defect of ssc1–2, correcting the translocation but not the folding defect. We found that although Ssc1 was competent to facilitate the refolding of denatured luciferase in vitro, both Ssc1–2 and Ssc1–201 showed significant defects, consistent with the data obtained with isolated mitochondria. Purified Ssc1–2 had a lowered affinity for a peptide substrate compared with wild-type Ssc1 but only in the ADP-bound state. This peptide binding defect was reversed in the suppressor protein Ssc1–201. However, a defect in the ability of Hsp40 to stimulate the ATPase activity of Ssc1–2 was not corrected in Ssc1–201. Thus, the inability of these two mutant proteins to efficiently facilitate luciferase refolding correlates with their defect in stimulation of ATPase activity by Hsp40s, indicating that this interaction is critical for protein folding in mitochondria.

Molecular chaperones such as members of the 70-kDa class (Hsp70s) bind to nonnative conformations of proteins, thus facilitating their folding and translocation across membranes (1, 2). The C-terminal 28-kDa region of Hsp70s binds unfolded polypeptides, whereas the highly conserved N-terminal 44-kDa domain regulates that binding through its interaction with adenine nucleotides (3). It is thought that Hsp70 proteins, like many GTPases, have a two-state conformation. When an ADP molecule is bound in the nucleotide-binding site, the Hsp70 exhibits relatively stable polypeptide substrate binding; when ATP is bound, binding of substrate is relatively unstable. The 44-kDa domain has a low intrinsic ATPase activity; therefore, ATP hydrolysis converts Hsp70 to a form having a relatively stable interaction with unfolded proteins. Exchange of ADP for ATP results in destabilization of the interaction. It is thought that a polypeptide first interacts with a Hsp70 in the ATP-bound state, and then hydrolysis of ATP to ADP stabilizes this interaction.

This cycle of interaction is facilitated by cochaperones. Prokaryotes and mitochondria contain Hsp40-type cochaperones as well as nucleotide release factors such as GrpE of Escherichia coli and Mge1 of Saccharomyces cerevisiae (4, 5). In the simplest scenario, nucleotide release factors are thought to destabilize the interaction of unfolded proteins with Hsp70, as release of ADP from a DnaK-ADP complex can be increased up to 5000-fold by GrpE action (4). However, the action of Hsp40s such as DnaJ of E. coli is less well understood. Hsp40s stimulate the ATPase activity of Hsp70s, which is thought to facilitate their binding to unfolded polypeptide substrates (6). Hsp40s contain a canonical J domain that interacts with the ATPase domain of Hsp70s (7). There may also be a site of interaction of Hsp40s with the C-terminal domain of Hsp70s, since mutant Hsp70s that show a defect in interaction with peptide substrates also show a defect in interaction with Hsp40s (8, 9).

In addition to interacting with Hsp70s, many Hsp40s, including E. coli DnaJ, bind unfolded or partially folded polypeptides, preventing their aggregation (10). Based on a variety of in vitro analyses, a model of how Hsp40s and Hsp70s cooperate in protein folding has evolved (2, 3). Polypeptide substrates first bind Hsp40. Then, the ATPase domain of Hsp70 interacts with Hsp40 via its J domain. This interaction not only brings the substrate in close proximity to Hsp70, but it also stimulates hydrolysis of ATP, trapping the substrate. Subsequent release of nucleotide and rebinding of ATP destabilize the Hsp70-substrate complex, resulting in its release.

In eucaryotes, all major cellular compartments contain at least one Hsp70 and one Hsp40. Ssc1, the major Hsp70 of the mitochondrial matrix, is involved in the translocation of proteins from the cytosol across the mitochondrial inner membrane to the matrix and their subsequent folding. Two temperature-sensitive mutants, ssc1–2 and ssc1–3, have been used extensively in the analysis of the physiological roles of Ssc1 (11–15). The ssc1–3 mutation causes an amino acid substitution in the ATPase domain that results in severe defects in translocation. The ssc1–2 mutation, which changes a single amino acid in the peptide binding domain (P442S) (12), has less severe effects and is therefore more useful in dissecting the roles of Ssc1 in both translocation and folding. Intragenic suppressors of ssc1–2, which cause an additional single amino acid alteration in the peptide binding domain of Ssc1–2, have been isolated. These suppressor mutations allow robust growth at the intermediate temperature of 34 °C but do not completely suppress the growth defect of ssc1–2 above that temperature. Two of these, Ssc1–201 and Ssc1–202 (D519E and V524I, respectively) have been analyzed (15). Both suppress the defects in protein translocation as well as the interaction with the membrane tether Tim44. However, initial results suggested that the folding defect is not suppressed (15).

To better understand the role of Ssc1 in protein folding within the mitochondrial matrix, we have undertaken a char-
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acetylation of the biochemical properties of Ssc1, Ssc1–2, and Ssc1–201. We found that neither the ATPase activity of Ssc1–2 nor of the suppressor protein Ssc1–201 was stimulated by Hsp40s. In addition, both show significant defects in their ability to facilitate the refolding of luciferase in vitro. These results make evident the importance of Hsp40s in facilitating protein folding in the mitochondrial matrix in vivo and provide insight into the complex interactions between Hsp40s and Hsp70s.

EXPERIMENTAL PROCEDURES

Plasmids and Strains

pRS314-SSC1(His tag) was constructed from pRS314-SSC1 (16) by inserting six codons encoding histidine at the 3′ end of the SSC1 gene using polymerase chain reaction. The segments of sscl–2, ssc1–201, and ssc1–202 encoding mutations were subcloned into this plasmid.

An expression plasmid (pGEM-Su9-DHFR*)1 that contained a mutant version of DHFR (C75S, S42C, and D49C), DHFR*, for use in import and folding assays was constructed. The Su9 and DHFR* portions were polymerase chain reaction-amplified from pGEM-Su9-DHFR (15) and DHFR*1 (17), respectively, using Pfu DNA polymerase (Stratagene, La Jolla, CA). A 3′ untranslated fragment of the final polymerase chain reaction product was inserted into pGEM-Su9-DHFR to replace the wild-type (WT) portion of DHFR. The plasmid was confirmed by DNA sequencing and restriction enzyme digests.

The yeast strain used for Ssc1 purification was obtained by crossing WY11 (his3–11, 15 leu2–3, 112 ura3–1 can1–100 GAL2::HYG* and trp1–1 ssc1–1 D::LEU2) with WY12 (his3–11, 15 ade2–1 can1–100 GAL2+ met2–1 leu2–3 112 his3–11, 15 ade2–1 can1–100 GAL2+ met2–1 leu2–3 trp1–1 ssc1–1 D::LEU2 pRS316-SSC1 (QL1). After transforming QL1 with pRS314-Ssu9-DHFR* (His tag) containing mutant or WT versions of SSC1, cells lacking pRS316-SSC1 were selected on 5-fluoroorotic acid plates. Various concentrations of Ssc1 proteins were incubated with F-P5 (10 mM) at 25 °C in buffer A (50 mM Hepes-KOH, pH 7.4, 150 mM KCl, 1 mm magnesium acetate, 10 mM glycerol) and refolded with low salt buffer (20 mM Hepes-KOH, pH 7.4, 10 mM KCl, 10% glycerol). After washing with low salt buffer, Ssc1 was eluted with high salt buffer (20 mM Hepes-KOH, pH 7.4, 200 mM KCl, 10% glycerol). The fractions containing Ssc1 protein were pooled, frozen in liquid nitrogen, and stored at −75 °C.

Mitochondria were isolated from wild-type and ssc1 mutant (ssc1–2 and ssc1–201) yeast strains grown at 25 °C in YPGlycerol media (1% yeast extract, 1% peptone, 0.5% glycerol, and 2% ethanol) as described previously (12, 15). Precursor proteins Su9-DHFR and Su9-DHFR* were synthesized in rabbit reticulocyte lysate in the presence of [35S]methionine. Before import, the precursor proteins were denatured by 7 M urea, and mitochondria were incubated at 37 °C for 15 min to induce the temperature-sensitive phenotype. The import reactions were stopped by the addition of valinomycin. Mitochondria were further incubated at 25 °C for various times then lysed with Triton X-100. After a clarifying spin, the extract was incubated with 2 mM ATP at 25 °C for 30 s in buffer A, then F-P5 was added. By 2 min, the reaction was stopped, and the percent conversion of ATP to ADP was determined. The rate of ATP hydrolysis was calculated by fitting the data to a first-order rate equation by nonlinear regression analysis using Prism 2.0.

In experiments utilizing ADP, Ssc1 was incubated with 500 μM nucleotide for 15 min in buffer A. Similar results were obtained with Ssc1 proteins with and without a 37 °C preincubation and in the absence of ADP. For the binding in the presence of ATP, Ssc1 proteins were incubated with 2 μM ATP at 25 °C for 30 s in buffer A, then F-P5 was added. By 2 min, significant hydrolysis occurred, binding equilibrium was reached, and anisotropy readings were taken. The release rate was calculated by fitting the release curve to one phase exponential decay using Prism 2.0 (GraphPad, San Diego, CA).

Mitochondrial Assays

Mitochondria were isolated from wild-type and ssc1 mutant (ssc1–2 and ssc1–201) yeast strains grown at 25 °C in YPGlycerol media (1% yeast extract, 2% peptone, 3% glycerol, and 2% ethanol) as described previously (12, 15). Precursor proteins Su9-DHFR and Su9-DHFR* were synthesized in rabbit reticulocyte lysate in the presence of [35S]methionine. Before import, the precursor proteins were denatured by 7 M urea, and mitochondria were incubated at 37 °C for 15 min to induce the temperature-sensitive phenotype. The import reactions and subsequent immunoprecipitations using Ssc1 antibodies were carried out as described (15). Briefly, after import for 2 min at 25 °C, the import reaction was stopped by the addition of valinomycin. Mitochondria were further incubated at 25 °C for various times then lysed with Triton X-100 buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.1% Triton X-100). After a clarifying spin, the extract was incubated with Ssc1 antibody cross-linked to protein A-Sepharose beads at 4 °C for 1 h. The imported proteins bound to the protein A-Sepharose beads were analyzed by SDS-polyacrylamide gel electrophoresis and digital autoradiography.

To assess folding of imported proteins, a 2-min import reaction was carried out. Mitochondria were then disrupted with 0.6% Triton X-100 and treated with protease K (15 μg/ml) (Roche Molecular Biochemicals) for 15 min on ice. After the digestion was stopped by the addition of phenylmethylsulfonyl fluoride, proteins were precipitated with 5% trichloroacetic acid and analyzed by SDS-polyacrylamide gel electrophoresis and digital autoradiography.

Protein Purification

The yeast strains QL2, QL3, QL4, and QL5 were grown at 25 °C in YPGlycerol media. Cells were harvested at A600 = 1.0–1.5, and mitochondria were isolated as described previously with some modifications (12). Briefly, after treatment with zymolase (0.45 mg/ml) (ICN Biomedicals, Costa Mesa, CA), the cells were homogenized in homogenization buffer 0.6 M sorbitol, 10 mM Tris-HCl, pH 7.5, and 1 mM phenyl-

1 The abbreviations used are: DHFR, dihydrofolate reductase; WT, wild type.
the synthesized protein was denatured with urea and imported into mitochondria isolated from wild-type, ssc1–2, and ssc1–201 cells. In agreement with previous results (15), this unfolded protein was imported into ssc1–2 as efficiently as into mitochondria from wild-type or ssc1–201 cells, as very similar amounts of radiolabeled protein were imported into the 3 mitochondria preparations (Fig. 1A). The folding state of the imported DHFR was assessed by testing its susceptibility to digestion by protease. After import, the isolated mitochondria were disrupted by Triton X-100, and the extracts were treated with protease K. 49% of imported DHFR was protease K-resistant in wild-type mitochondria, whereas only 17% was resistant in ssc1–2 mitochondria. 23% of imported DHFR was resistant in ssc1–201 mitochondria. Therefore, both ssc1–2 and ssc1–201 mitochondria are defective in the folding of this test substrate. However, DHFR imported into ssc1–201 mitochondria consistently showed slightly more protease K resistance than that in ssc1–2 mitochondria.

Since we observed a defect in folding in ssc1–2 and ssc1–201 mitochondria, we wanted to test the ability of the mutant proteins to function in the refolding of denatured proteins in vitro. First we developed a purification scheme for wild-type and mutant Ssc1 proteins. We utilized an SSC1 gene encoding a C-terminal polyhistidine extension. These constructs rescued the lethality of the ssc1 mutation, and strains carrying His-tagged mutant versions maintained the same temperature-sensitive growth phenotype as those carrying untagged genes (data not shown).

Under the conditions used, refolding of denatured luciferase was dependent upon the presence of Ssc1, the mitochondrial Hsp40 Mdj1, and the nucleotide exchange factor Mge1 as well as Hsp78, a member of the Hsp100 family of chaperones that facilitates disaggregation of protein aggregates (22, 23). In the absence of Ssc1, less than 1% of luciferase activity was recovered, whereas in the presence of Ssc1, ~60% of luciferase activity was found as compared with the native luciferase (Fig. 2A). However, Ssc1–2 was not able to significantly facilitate the refolding of luciferase under any condition tested. In addition, no activity of Ssc1–201 was observed at low concentrations of Ssc1, where the refolding activity of wild-type Ssc1 is nearly maximal (1 μM). However, at increasing concentrations, some refolding activity was observed, reaching about 45% maximal wild-type activity at 6 μM. Therefore, although the suppressor protein has some refolding activity, it is severely compromised compared with wild-type protein. This result is consistent with the slightly increased level of resistance of imported DHFR to protease in ssc1–201 mitochondria compared with ssc1–2 mitochondria, even though the level of Ssc1 protein is similar in all three types of mitochondria (data not shown).

**Ssc1–2 and Ssc1–201 Have a Prolonged Interaction with Imported Protein—Ssc1 interaction with imported proteins can be monitored in mitochondria by testing for the ability of Ssc1-specific antibodies to coimmunoprecipitate radiolabeled imported protein. In wild-type mitochondria, this association is transient. Consistent with previously published results (15, 24), only a low percentage of imported protein, about 5%, was immunoprecipitated immediately after import (Fig. 3A). With time, that amount decreased, to 1–2% by 15–35 min after import. Significantly larger amounts of DHFR were coimmunoprecipitated in ssc1–2 and ssc1–201 mitochondria. Initially 12–15% was precipitated; by 35 min after import, 7–9% was precipitated.

Thus, ssc1–2 and ssc1–201 mitochondria showed a defect in folding of DHFR and a prolonged association with imported protein. Two possible explanations for the prolonged association came to mind. First, Ssc1–2 and Ssc1–201 might have an increased affinity for unfolded proteins. Perhaps the substrates are released more slowly once they have bound to the mutant Hsp70, causing this prolonged association. Alternatively, the mutant Hsp70s might have normal interaction with unfolded proteins but be defective in other interactions, resulting in a defect in folding. Proteins would thus remain in a partially unfolded state for a long period of time and remain substrates for Ssc1 binding. In this case each DHFR molecule would undergo many more cycles of interaction with mutant Ssc1 proteins, causing an apparent prolonged interaction. We proceeded to test these ideas.

**Ssc1–2, but Not Ssc1–201, Has a Lowered Affinity for Peptide Substrate in the ADP-bound Form but Not the ATP-bound Form.** To examine substrate binding properties, wild-type, Ssc1–2, and Ssc1–201 proteins were tested in a fluorescence
anisotropy-based peptide binding assay. This assay has been previously used to assess the interaction of peptide substrates with DnaK (9, 21). For studies of Ssc1, a model peptide P5 (CALLLSAPRR), having a portion of the mitochondrial-targeting sequence of aspartate aminotransferase from chicken, was selected. P5 was fluorescently labeled on its N-terminal cysteine with fluorescein (F-P5). The anisotropy assay follows the relative rotational diffusion of the fluorescein after excitation with polarized light. Because of its small size, F-P5 should rotate in solution rather rapidly and, thus, have a low anisotropy value. When F-P5 is bound to Ssc1, it should rotate more slowly and, thus, display a significantly higher anisotropy value.

Binding assays were performed using increasing concentrations of Ssc1 protein, and the increase in anisotropy was fitted to a single-site binding model. Analysis of wild-type Ssc1 binding experiments, carried out in the presence of ADP, yielded a dissociation constant ($K_d$) of 0.22 ($\pm$ 0.036) $\mu$M (Fig. 4, A and B). Ssc1–2 showed a 5-fold lower affinity for peptide, having a $K_d$ of 1.1 ($\pm$ 0.095) $\mu$M. However, the affinity of Ssc1–201 for peptide was only slightly lower than that of wild-type, having a $K_d$ of 0.28 ($\pm$ 0.031) $\mu$M. Therefore, the suppressor mutation appears to have significantly reversed the defect of Ssc1–2 in binding peptide substrate.

To look at the defect of Ssc1–2 in more detail, we monitored the displacement of the prebound F-P5 after the addition of a large excess of unlabeled P5 by measuring the decrease in anisotropy. Upon the addition of P5 to a wild-type Ssc1-F-P5 complex, the anisotropy change showed a single phase with a rate constant of 0.0024 ($\pm$ 0.000022) s$^{-1}$ (Fig. 4C). Experiments with Ssc1–2 revealed a very similar $k_{on}$ (0.0028 ($\pm$ 0.000059) s$^{-1}$). This small difference in $k_{on}$ observed using ADP-bound Ssc1–2 cannot account for the 5-fold difference in $K_d$ observed. Therefore, we conclude that this difference in $K_d$ is due to a difference in the on-rate of peptide. Similar values were obtained when the experiments were performed in the absence of nucleotide.

We also tested the suppressor protein Ssc1–201 to determine the basis of the restoration of the $K_d$ to wild-type levels. The $k_{on}$ was decreased about 2.2-fold compared with wild-type protein, 0.0011 ($\pm$ 0.0000062) s$^{-1}$ compared with 0.0024 s$^{-1}$. Therefore, to obtain a $K_d$ similar to wild-type, the $k_{off}$ of Ssc1–201 would need to be 2.8-fold slower than that of wild type. We proposed that both the $k_{on}$ and $k_{off}$ are affected by the suppressor mutation, resulting in a nearly normal affinity.

The affinity of Hsp70 for peptide substrates is much lower in the ATP-bound form than in the ADP-bound form (25, 26). As expected, upon the addition of ATP, the anisotropy readings rapidly decreased, indicating peptide release. Because of the rapidity of this reduction, we were unable to compare the rate of release of peptide upon ATP addition among the different proteins.

To look more carefully at the interaction of Ssc1 with peptide when it is in the ATP-bound state, we carried out anisotropy assays in the presence of ATP. As expected, in the presence of ATP, the affinity for peptide of both wild-type Ssc1 and Ssc1–2 was dramatically reduced (Fig. 4A). Because of this reduction we were unable to approach saturation of binding. However, the affinity of wild-type Ssc1 and Ssc1–2 appears similar, based on the increase in anisotropy observed at high concentrations of protein. We estimate that the $K_d$ of both wild-type Ssc1 and Ssc1–2 proteins in the ATP-bound form is on the order of 20 $\mu$M, if at saturation the anisotropy reading is similar to that of the ADP-bound form. Therefore, there is about a 90-fold difference between the affinity of the ATP and ADP form of wild-type protein for P5. This difference is similar to the difference in affinity of ATP and ADP-bound DnaK for peptides (25, 27, 28). In summary, our results indicate that wild-type Ssc1 and Ssc1–2 have similar affinity for peptide substrate in the presence of ATP. However, in the presence of ADP, the mutant Ssc1–2 protein has a lower affinity than wild-type protein. The suppressor mutation in ssc1–201 substantially corrects this decreased affinity found in the ssc1–2 protein.

**Unfolded DHFR Has a Prolonged Association with Both Wild-type and Mutant Ssc1 Proteins**—Since the analysis of purified proteins provided no indication that the mutant proteins had a higher affinity for peptide substrates, we tested the idea that the failure of DHFR to fold in the mitochondrial matrix would result in a prolonged association of wild-type Ssc1. We took advantage of a previously characterized mutant of mouse DHFR (DHFR*), which encodes three structurally destabilizing amino acid alterations (29). These amino acid changes result in an increased susceptibility to protease and diminish the ability of methotrexate, which stabilizes the structure of wild-type DHFR to block import into mitochondria (17). Upon import into mitochondria the mutant DHFR was extremely susceptible to digestion with protease (Fig. 1B). In addition, 30–40% of the mutant DHFR could be immunoprecipitated using Ssc1-specific antibodies, with only a slight decrease over time (Fig. 3B). Therefore a protein known to be defective in folding has a prolonged association with both mutant and wild-type Ssc1. We conclude that the prolonged inter-
action observed with Ssc1–201 (and Ssc1–2) mitochondria is a direct result of the failure of DHFR to fold, thus remaining a substrate for extended periods of time.

The ATPase Activity of Both Ssc1–2 and Ssc1–201 Are Stimulated Normally by Peptide—Hsp70s have two basic activities: binding hydrophobic peptide segments and ATPase activity. Since the peptide binding ability of the mutant proteins does not appear to explain the protein folding defect of Ssc1–2 and Ssc1–201, we analyzed ATPase activities. For this purpose we used a single turnover assay, monitoring the hydrolysis of prebound radiolabeled ATP. The ATPase activity of the three proteins differed less than 2-fold. Wild-type Ssc1 had a rate constant of 0.063 (±0.0039) min⁻¹, whereas the observed rate constants for Ssc1–2 and Ssc1–201 were 0.10 (±0.019) and 0.12 (±0.014) min⁻¹. This rate is similar to that found for other Hsp70s (30).

Binding of peptide stimulates the rate of ATP hydrolysis of Hsp70s (31, 32). Therefore, we compared stimulation of wild-type Ssc1 and the mutant proteins by P5 over a range of concentrations. As can be seen in Fig. 5A, the ability of peptide to stimulate the hydrolysis of ATP by the wild-type and mutant proteins is similar. This result is consistent with the anisotropy results described above, indicating that Ssc1–2 and wild-type protein have a similar affinity for peptide in the ATP-bound form.

Hsp40 Stimulation of the ATPase Activity of Both Ssc1–2 and Ssc1–201 Are Defective—Ssc1 functions with co-chaperones Mge1 and Mdj1 (33–35). To assess the nucleotide release activity of Mge1, we used a single turnover ATPase assay, monitoring the hydrolysis of radiolabeled ATP prebound to Ssc1. This assay is based on the idea that release of nucleotide from Ssc1 facilitated by Mge1 will cause a decrease in hydrolysis of the radiolabeled ATP. Since an excess of unlabeled ATP is included in the reaction, released radiolabeled nucleotide will only rarely rebind Ssc1. As previously published (5, 16), the addition of nonradioactive ATP to the reaction had little effect on the hydrolysis of the prebound ATP, indicating that the
nucleotide remains bound throughout the time course of the reaction (data not shown). However, as previously reported, the addition of wild-type Mge1 reduced the hydrolysis of radiolabeled ATP when unlabeled nucleotide was added to prevent rebinding of released radiolabeled nucleotide (Fig. 6). In the presence of Mge1 and unlabeled ATP, ADP formation reached a plateau at only 10% hydrolysis. Very similar results were obtained with Ssc1–2 and Ssc1–201, indicating that these mutant proteins were not defective in their interaction with Mge1.

To assess the functional interaction of Hsp40s with Ssc1, we again utilized the single turnover ATPase assay, as it is well established that Hsp40s stimulate the hydrolysis of ATP by Hsp70s (32, 36). We observed a 6-fold stimulation of wild-type Ssc1 upon the addition of Mdj1 at a concentration of 4 μM; DnaJ stimulated Ssc1 more than 12-fold at the same concentration (Fig. 5, B and C). However, at the same concentration, Mdj1 did not measurably stimulate the ATPase activity of either Ssc1–2 or Ssc1–201, whereas DnaJ stimulated less than 2-fold. Thus, both Ssc1–2 and the suppressor protein Ssc1–201 are defective in their interaction with Hsp40s. In addition, a second suppressor, Ssc1–202, was purified and analyzed. In this, and other assays described above, Ssc1–202 behaved very similarly to Ssc1–201 (data not shown).

We tested the effect of increasing concentrations of Mdj1 in in vitro assay to determine whether increasing the concentration of Mdj1 would overcome the folding defect of Ssc1–2 or Ssc1–201. However, at higher concentrations, Mdj1 was inhibitory, even in the case of wild-type Ssc1 (Fig. 2B). Perhaps at higher concentrations more Mdj1 binds to the denatured luciferase, perhaps inhibiting aggregation but inhibiting a productive folding reaction as well.

FIG. 6. ATP release activity of Mge1 on Ssc1 proteins in single-turnover ATPase assays. Ssc1-ATP complex (~1 μM) was incubated at 25 °C in the presence or absence of Mge1 (6 μM) and in the presence of 250 μM ATP. Samples were withdrawn at the indicated times, and the fraction of ATP converted to ADP was determined. Open circles, no Mge1 added; closed circles, Mge1 added. A, WT Ssc1. B, Ssc1–2. C, Ssc1–201.

Our goal in this study was to better understand the mechanism of action of molecular chaperones in protein folding in the mitochondrial matrix. Both mutant Ssc1 proteins studied here, Ssc1–2 and Ssc1–201, are defective in protein folding in isolated mitochondria as well as in in vitro folding assays. In addition, both are impaired in their functional interaction with Hsp40, suggesting not only a requirement for Hsp40 in the mitochondrial matrix for folding of some proteins but also a requirement for an interaction between the two chaperones. This idea is in agreement with previous analyses, as mitochondria lacking Mdj1 are defective in the folding of imported DHFR (37). However, even though an interaction between Mdj1 and Ssc1 has been detected in isolated mitochondria (35), little is understood about its importance.

Several studies of the E. coli system indicate the importance of a physical interaction between DnaK and DnaJ. But the nature of the interaction between Hsp40 and Hsp70 is complex and not fully understood. Evidence suggests an interaction between DnaJ and both the ATPase and peptide binding domains of Hsp70. The interaction between the J domain and the Hsp70 ATPase domain is well established. NMR studies revealed such an interaction (7), and amino acid alterations in a groove of the DnaK ATPase domain resulted in a decrease in DnaJ interaction and a decrease ability to refold luciferase in vitro (38). In addition, an amino acid alteration on the same face of the ATPase domain of DnaK as this groove was found to suppress not only the temperature-sensitive in vitro defect caused by an alteration in the J domain of DnaJ but also the defective physical interaction between the mutant DnaJ and DnaK (8).

The amino acid alterations in Ssc1–2 and Ssc1–201 occur in the peptide binding domain. An interaction between Hsp40 and the peptide binding domain of Hsp70 occurs in vitro. However, the question of the biological relevance of this interaction remains open. Several dnaK mutant proteins having amino acid alterations in the peptide binding domain resulting in a lowered affinity for peptide substrates have been tested for DnaJ interactions. All tested mutants have an impaired interaction with DnaJ (8, 9, 21, 39). Several issues are unresolved. It remains unclear whether in vitro Hsp40 binds a substrate in the peptide binding cleft or if there is a unique (or overlapping) site in the C terminus at which a Hsp40 binds. Also, the interaction of Hsp40s with the peptide binding region may be an in vitro artifact, not a biologically relevant interaction. At any rate, for in vitro detection of most Hsp40-Hsp70 interactions, an interaction of the Hsp40 at or near the peptide binding cleft of Hsp70 appears to be required.

Ssc1–201 is unusual in that it is defective in interaction with Hsp40s even though interaction with peptide substrates appears normal, as measured by affinity for peptide and the ability of peptide to stimulate hydrolysis of Hsp70-bound ATP. Therefore, for the first time, an amino acid alteration in the peptide binding domain separates peptide binding from interaction with Hsp40. Two different explanations could account
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for this separation. First, this alteration could be revealing the site of interaction with Hsp40 that is distinct (or perhaps overlapping) with the site of interaction with peptide. If so, our results suggest that the interaction of Hsp40 with the peptide binding domain is biologically meaningful. Alternatively, the alterations might alter the interaction between the ATPase and peptide binding domains such that the interaction of the J domain with the N terminus is affected. There are several arguments against this second possibility as some aspects of communication between the domains are normal in both Ssc1–2 and Ssc1–201. Peptide binding stimulates the ATPase activity of the N-terminal domain normally, and binding of ATP results in the rapid dissociation of peptide substrates. In addition, both Ssc1–2 and Ssc1–201 seem able to undergo normal conformational changes in response to nucleotide binding as measured by tryptophan fluorescence (data not shown). At the moment, technical limitations prohibit us from definitively discriminating between these two alternatives. However, mutants such as ssc1–201 may be useful in dissecting the importance of the interaction of Hsp40s with the peptide binding domain of Hsp70 in the future.

An interesting characteristic of the Ssc1–2 protein is its altered binding to peptide substrates. Our data indicate that peptide binding is normal in the ATP but not the ADP-bound form. In the ADP-bound form, Ssc1–2 has a 5-fold lower affinity for a peptide substrate. This reduced affinity is mainly due to a decreased on-rate. However, interaction with the ATP form appears normal, as indicated by stimulation of ATPase by peptides and the association of fluorescent peptide with the ATP-bound form of the protein. Extensive work by Bukau and co-workers (27) has recently shown a positive correlation between the affinity of binding of substrates to ADP-bound DnaK and the ability of these substrates to stimulate ATPase activity. This correlation held regardless of whether DnaK lacked the α-helical lid over the peptide binding domain or had alterations in either the “arch” over the peptide binding cleft or in the cleft itself (27). Our preliminary analysis with alterations in the peptide binding domain of Ssc1 has indicated a similar correlation; a decrease in affinity for peptide substrates in the ADP-bound form should be inconsequential, as binding occurs only in the ATP-bound form, which is then converted to the high affinity Hsp70-ADP form by stimulation of hydrolysis. Our data raise the question as to whether the binding to substrate in the ADP-bound form has some physiologically role. In fact, the ssc1–201 suppressor mutation does allow some recovery of in vitro folding activity although still far from wild-type levels. The mutants studied here as well as others isolated in the future having similar differential effects on the interaction of substrates with the two nucleotide-bound forms should allow this question to be addressed.

In summary, our results suggest that the defect in protein folding in the mitochondrial matrix of a Hsp70 mutant is due to a defective interaction with Hsp40. Such comparisons between defects in the complex milieu of the mitochondria and analysis of biochemical defects found in purified systems will be important in understanding the mechanism of action of molecular chaperones in complex physiological processes such as protein translocation and folding.

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