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Ssq1, a Mitochondrial Hsp70 Involved in Iron-Sulfur (Fe/S) Center Biogenesis

SIMILARITIES TO AND DIFFERENCES FROM ITS BACTERIAL COUNTERPART*

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The results of *in vivo* and *in organellar* experiments indicate that the Hsp70 Ssq1 and the J-protein Jac1 function together to assist in the biogenesis of iron-sulfur (Fe/S) centers in the mitochondrial matrix. Here we present biochemical evidence supporting this idea. Isu, the proposed scaffold on which Fe/S centers are assembled, is a substrate for both Jac1 and Ssq1. Jac1 and Isu1 cooperatively stimulate the ATPase activity of Ssq1. In addition, Jac1 facilitates the interaction of Ssq1 with Isu1 in the presence of ATP. These findings are consistent with the role in Fe/S biogenesis previously proposed for the bacterial Hsp70 Hsc66 and J-protein Hsc20 that interact with the bacterial Isu homologue IscU. However, unlike the bacterial Hsp70, we found that Ssq1 has a high affinity for nucleotide, and shares a nucleotide exchange factor, Mge1, with a second mitochondrial Hsp70, Ssc1. Thus, whereas the bacterial and mitochondrial chaperone systems share critical features, they possess significant biochemical differences as well.

Extensive genetic and cell biological evidence supports the idea that two mitochondrial molecular chaperones work together, along with other proteins, in the processes of iron-sulfur center biogenesis and mitochondrial iron homeostasis (1, 2). These chaperones, the Hsp70 Ssq1, and the J-protein Jac1 are localized to the mitochondrial matrix (3–5). Mutation of the gene encoding either of these chaperones results in a substantial decrease in the activities of mitochondrial proteins containing Fe/S centers, including aconitase, the cytochrome bc_1 complex, and succinate dehydrogenase, as well as a significant increase in mitochondrial iron level (4–8). Mitochondrial proteins functioning in biogenesis of Fe/S centers are related to bacterial proteins encoded by the *isc* (iron-sulfur cluster) operon, indicating a bacterial origin of this important process (for review, see Ref. 9). Among the proteins encoded by the *isc* operon of *Escherichia coli* are two chaperones, Hsc66, an Hsp70 and Hsc20, a J-type protein (10, 11), along with IscU, the protein that is thought to serve as a scaffold on which Fe/S centers are built prior to transfer to a recipient protein (12).

Hsc66 shares many biochemical properties with other Hsp70s, including another Hsp70 of the bacterial cytosol, the abundant and well studied DnaK. Both possess ATPase activities, which are stimulated by specific J-type co-chaperones: DnaJ for DnaK and Hsc20 for Hsc66 (13). Although Hsp70 and J-proteins most often bind unfolded or partially folded polypeptides, a few native proteins have been identified as substrates. For example, the transcription factor σ32 and phage λ DNA replication proteins λP and λO are substrates of DnaK and DnaJ (14). Recent evidence indicates that IscU, the scaffold for Fe/S center assembly, is a native substrate of the Hsc66/Hsc20 pair (15). As expected for a substrate protein, IscU stimulates the ATPase activity of Hsc66. In addition, the apparent affinity of Hsc66 for IscU is higher in the presence of ADP compared with ATP. This nucleotide sensitivity is a hallmark of an Hsp70 cycle of protein substrate binding and releasing (16). When ADP is bound, Hsp70 exhibits stable substrate binding; when ATP is bound, binding of substrate is unstable.

Whereas the ATP state of an Hsp70 is a low affinity state for substrate protein, the on-rate for substrate is very rapid, and thus thought to be the productive state for initiating binding *in vivo*. ATP hydrolysis then promotes conversion to the high affinity state, and ADP release, followed by ATP binding, promotes substrate release, completing the chaperone cycle. J-proteins, because they both can bind protein substrates themselves and stimulate the ATP hydrolysis of Hsp70s have been proposed to "target" substrate proteins to Hsp70s (16). IscU binds Hsc20, as well as Hsc66, and stimulation of the ATPase of Hsc66 is enhanced several hundredfold when IscU and Hsc20 are present simultaneously. Thus, Hsc20 could well play a role in "targeting" of IscU, similar to the targeting of σ32 to DnaK by DnaJ (17, 18)

Transient interactions between Hsp70 and its protein substrates are essential for the biological functions of chaperones. If the off-rate of nucleotide from an Hsp70 is slow, a factor is needed to facilitate nucleotide release, which in turn promotes release of protein substrate. The DnaK/DnaJ chaperone system requires the nucleotide release factor GrpE (19). Only in the presence of both DnaJ and GrpE is the steady-state ATPase activity of DnaK significantly stimulated. In contrast, the off-rate of nucleotide from Hsc66 is much faster than that from DnaK (20, 21). Consistent with this observation, significant stimulation of Hsc66 ATPase activity by Hsc20 and IscU is observed in the absence of any nucleotide release factor (17). These data also imply that two distinct Hsp70 systems exist in *E. coli*: DnaK, functioning with co-chaperones DnaJ and GrpE and Hsc66 functioning with Hsc20.

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As in the bacterial cytosol, the mitochondrial matrix contains another Hsp70 system that is closely related to the DnaK/DnaJ/GrpE chaperone machine: the Hsp70 Ssc1, the J-protein Mdj1, and the nucleotide-release factor Mge1. Like the DnaK/DnaJ/GrpE system, these abundant proteins function generically, interacting with a wide variety of protein substrates. The DnaK system facilitates the folding of many proteins, particularly after periods of stress. The Ssc1 system engages in a wide spectrum of biochemical processes including protein translocation across the mitochondrial inner membrane, folding of proteins after import, and reactivation or degradation of proteins that either fold improperly or become permanently damaged after exposure to stress (for review see Ref. 22–24). However, both Hsc66 and Ssq1 are thought to function in a single metabolic process, the biogenesis of Fe/S centers. Thus far an important difference between these bacterial and mitochondrial Hsp70s has been determined. Whereas Hsc66 and DnaK are present at similar concentrations in bacteria (25, 26), Ssq1 is between 250- and 1000-fold less abundant than Ssc1, which constitutes ~2% of mitochondrial protein (7).

Whereas there is much *in vivo* evidence to suggest that Ssq1 and Jac1 function together in Fe/S center biogenesis, no direct biochemical evidence has been presented. Here we report that Ssq1 functions with Jac1 by stimulating binding of Ssq1 to its specific native protein substrate, Isu1, a homolog of bacterial IscU. We also show that in contrast to bacterial Hsc66, mitochondrial Ssq1 stably binds ATP and, therefore, requires a nucleotide release factor, Mge1, for stimulation of its ATPase activity. Thus, although there are significant similarities in the biochemical properties of Ssq1 and Hsc66, there are significant differences as well.

EXPERIMENTAL PROCEDURES

Overexpression and Purification of Proteins—Recombinant $Jac1_{His}$ was purified from yeast cells as described (4). It was judged to be 98% pure by SDS-PAGE and staining with Coomassie Blue. Recombinant $Mge1_{His}$ and $Mdj1_{His}$ were purified from *E. coli*, as described (27) and were 95 and 90% pure, respectively. Hsc66 and IscU were a kind gift of Dr. L. E. Vickery (University of California, Irvine, CA). Yfh1, lacking its presequence, was purified from *E. coli* and judged to be 98% pure (a kind gift of Dr. K. Aloria, University of Wisconsin, Madison, WI).

To construct an expression vector for purification of the Ssq1 protein, six histidine codons were introduced at the 3′ end of the *SSQ1* protein coding region by PCR. The PCR product containing the entire *SSQ1* coding sequence tagged with six histidine codons was cloned under the control of galactose-inducible promoter using the pYES2.0 vector (Invitrogen). $Ssq1_{His}$ was purified from a yeast strain defective in proteinase A, harboring the pYES2$Ssq1_{His}$ plasmid, after growth for 16 h on minimal drop-out media without uracil containing 2% galactose. Harvested cells were treated with Zymolase 20T (ICN) as described in Ref. 28. Spheroplasts were lysed in a French Press at 16,000 p.s.i. in buffer L (20 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.5 M NaCl, 0.5% (v/v) Triton X-100, 20 mM imidazole, pH 8.0). After a clarifying spin, the supernatant was loaded on a Ni^{2+}-NTA[1] column (Qiagen) and after washing with buffer L (50 column volumes), protein was eluted by a 20–350 mM linear imidazole gradient in buffer L (15 column volumes). Fractions containing Ssq1 were collected and dialyzed overnight in buffer P (20 mM potassium phosphate, pH 6.8, 150 mM KCl, 10% glycerol (v/v), 5 mM β-mercaptoethanol, 0.05% Triton X-100), then loaded on a hydroxylapatite (Bio-Rad) column equilibrated with buffer P. After washing with 10 column volumes, protein was eluted with a linear potassium phosphate gradient from 20 to 220 mM in buffer P (20 column volumes). Fractions containing Ssq1 were dialyzed in buffer P and applied onto a small Ni-NTA column equilibrated with buffer P. Ssq1 was eluted with buffer P containing 0.35 M imidazole, pH 6.8. Purified Ssq1 was dialyzed to buffer K (20 mM Tris-HCl, pH 8.0, 10% glycerol (v/v), 5 mM β-mercaptoethanol, 0.05% Triton X-100, 200 mM KCl) and stored in −70 °C. The final protein preparation was at least 85% pure as judged by the SDS-PAGE and staining by Coomassie Blue. It was not contaminated by visible amounts of unprocessed precursor form. The construction of an expression vector and the purification of $Isu1_{His}$ protein from yeast cells followed the same strategy described above for Ssq1, except protein having only been subjected to chromatography on a Ni-NTA column, which was 90% pure, was used for N-terminal sequencing as described for Jac1 (4).

To construct an expression vector for purification of $Isu1_{His}$ from *E. coli*, the protein coding region lacking the first 35 codons (presequence determined by N-terminal sequencing) and containing six histidine codons at the 3′ end were amplified by PCR from the pYES2$Isu1_{His}$ plasmid. This PCR product was used to construct plasmid pET21d-$Isu1_M$. Expression of Isu1 was induced in the *E. coli* strain C41 (29) by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside at A_{600} = 0.6. After 3 h, cells were harvested and lysed in a French Press in buffer L containing 50 mM imidazole, pH 8.0, as described for Ssq1. After a clarifying spin, the supernatant was loaded on a Ni-NTA column equilibrated in buffer L. Proteins were eluted with a 50–500 mM imidazole gradient in buffer L (20 column volumes). Fractions containing Isu1, which were 95% pure, were collected, dialyzed against buffer K, and stored at −70 °C. The biochemical properties of $Isu1_{His}$ purified from yeast and bacterial cells were undistinguishable.

$Nfu1_{His}$ protein was overproduced in yeast cells harboring the vector pYES2$Nfu1_{His}$ constructed using the same strategy as for the Ssq1 and Isu1 proteins. The purification procedure of $Nfu1_{His}$ was the same as described above for Isu1. The purity of the final Nfu1 preparation was estimated to be 90%.

All His-tagged proteins used in this study were able to functionally replace untagged protein. Functionality was tested by constructing strains in which the only copy of a gene encoding a particular protein was a His-tagged version harbored on the low copy plasmid pRS316 (30). Growth of such strains was indistinguishable from their wild-type derivatives in medium containing different carbon sources at different temperatures (data not shown). Protein concentrations, determined using the Bradford (Bio-Rad) assay system using bovine serum albumin as a standard, are expressed as the concentration of monomers.

Complex Formation and Single Turnover Experiments—Ssq1·[α-^{32}P]ATP complex was formed and purified according to the published procedure (31) with some modifications. In short, Ssq1 protein (25 μg) was incubated with [α-^{32}P]ATP (10 μCi, DuPont NEG-003H, 3000 Ci/mmol) in 100 μl of buffer S (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, and 100 μM ATP) at 0 °C for 10 min. The reaction mixture was loaded on a gel filtration column (NICK, Amersham Biosciences) pre-equilibrated with buffer S at 4 °C. 80-μl fractions were collected. Fractions containing the first peak of radioactivity corresponding to the Ssq1·[α-^{32}P]ATP complex were pooled, adjusted to 10% (v/v) glycerol, aliquoted, and stored at −70 °C. An analogous procedure was used to obtain radioactive HscA·[α-^{32}P]ATP complex.

For single turnover experiments, 5 μl of Ssq1·[α-^{32}P]ATP or HscA·[α-^{32}P]ATP complexes was added to 15 μl of buffer S on ice containing various factors as indicated in the figure. legends, and incubated at 25 °C. At the indicated time points, 3 μl of the reaction mixture were 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 on PEI-cellulose TLC plate (Sigma) and developed in 1 M formic acid and 0.5 M LiCl. The TLC plate was exposed to a PhosphorImager system (Amersham Biosciences). Rates of ATP hydrolysis were calculated by fitting kinetic data to single exponential rise to maximum using nonlinear regression as described in Ref. 20.

In the experiments where Ssq1·[α-^{32}P]ATP complex was refractionated on a NICK (Amersham Bioscience) gel filtration column, the Ssq1·[α-^{32}P]ATP complex was incubated as described above for single turnover at 25 °C for 10 min to achieve 50–60% of ATP hydrolysis. Then, Mge1 was added at the indicated concentrations and the contents of the reaction were loaded onto a gel filtration column pre-equilibrated in buffer S at 4 °C. 80-μl fractions were collected. Aliquots from each fraction were mixed with stop solution and developed on PEI-cellulose as described above, to determine the relative amount of ATP and ADP. 50 μl of each fraction was also counted in liquid scintillation counter to determine the amount of radioactivity associated with each fraction.

Surface Plasmon Resonance (SPR) Analysis—SPR studies were carried out at 25 °C with a Biacore 2000 instrument (Piscataway, NJ). Purified proteins (Isu1, Mge1) were randomly cross-linked to the surface of the sensor chip CM5 by amine coupling as recommended by the manufacturer. Binding experiments were conducted in buffer R (25 mM HEPES-KOH, pH 7.5, 200 mM KCl, 11 mM $MgCl_2$, 0.005% (v/v) surfactant P20 (Amersham Biosciences)) containing 2 mM ATP when indi-

[1] The abbreviations used are: Ni-NTA, nickel-nitrilotriacetic acid; SPR, surface plasmon resonance.

cated, with the running buffer at a flow rate of 15 μl/min. 80 μl of buffer R containing the indicated purified proteins (Jac1, Ssq1) was used for injections.

Glycerol Gradient Centrifugation—Glycerol gradient centrifugation was carried out as described (32). Purified proteins (Isu1, Jac1, and Ssq1), alone or in combinations, were placed in reaction mixtures (80 μl) in buffer G (40 mM HEPES-KOH, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 10 mM $MgCl_2$, 5% (v/v) glycerol) containing 2 mM ATP or 2 mM ADP, and incubated for 10 min at 25 °C. Then, 70 μl of this mixture was loaded onto a 3-ml linear 15–35% (v/v) glycerol gradient prepared in buffer G with 2 mM ATP or 2 mM ADP as indicated, and centrifuged at 2 °C in a Beckman SW60 rotor for 28 h at 46,000 rpm. Fractions (130 μl each) were collected from the top of the tube and their protein contents were analyzed by SDS-PAGE followed by silver staining.

Steady-state ATPase Activity of Ssq1—The release of radioactive inorganic phosphate from [γ-^{32}P]ATP was measured as described (33) with minor modifications. Reaction mixtures contained Ssq1 and other proteins when indicated in buffer A (40 mM HEPES-KOH, pH 7.5, 100 mM KCl, 10% (v/v) glycerol, 1 mM dithiothreitol, 10 mM $MgCl_2$). Reactions were initiated by the addition of ATP (2 μCi, DuPont NEG-003H, 3000 Ci/mmol) to final concentrations as indicated in the figure legends. Incubation was carried out at 25 °C and terminated at the indicated time points by the removal of a 20-μl aliquot to an Eppendorf tube containing 175 μl of 1 M perchloric acid and 1 mM sodium phosphate. After addition of 20 mM ammonium molybdate (400 μl) and isopropyl acetate (400 μl), samples were vigorously mixed and the phases were separated by a short centrifugation. An aliquot of the organic phase (150 μl), containing the radioactive orthophosphate-molybdate complex, was removed and radioactivity was determined by liquid scintillation counting. Control reactions lacking protein were included in all experiments.

RESULTS

Isu1 Is a Native Substrate for the Ssq1/Jac1 Chaperone System—To begin to test the prediction that Jac1 is a co-chaperone of Ssq1, we measured the effect of Jac1 on the ATPase activity of Ssq1 using single turnover conditions. In this assay, a complex of purified Ssq1 protein with radioactive ATP was preformed and subjected to sizing chromatography to separate it away from free nucleotides. Then, the conversion of Ssq1/ATP to Ssq1/ADP with time was determined. This approach allowed measurement of the rate of ATP hydrolysis by Ssq1, unaffected by either the rate of ATP binding to, or the rate of dissociation of the reaction products from, Ssq1. ATP hydrolysis in the presence of different concentrations of Jac1 was then determined (Fig. 1, *A* and *B*). At a 1:1 ratio of Jac1:Ssq1, no stimulation of the basal activity was observed. At higher concentrations of Jac1, stimulation was observed. However, a vast excess of Jac1 was required to effect substantial stimulation. For example, a 50-fold excess of Jac1 resulted in a 4.8-fold stimulation.

Typically, binding of substrate polypeptides stimulates the ATPase activity of Hsp70s. For example, IscU protein, a native substrate of Hsc66, modestly stimulates its ATPase activity (17). We decided to search for potential substrates of Ssq1 by testing the ability of proteins linked to Fe/S center biogenesis and mitochondrial iron homeostasis to stimulate its ATPase activity. In addition to Isu1, the mitochondrial orthologue of bacterial IscU, and IscU itself, which has >60% identity with Isu1 (34), we tested two other yeast mitochondrial proteins, Nfu1 and Yfh1. None of these proteins affected Ssq1 ATPase activity even when present in 100-fold molar excess (Fig. 1*C*).

Because in other systems a native substrate and J-protein cooperatively stimulate the ATPase activity of Hsp70, we tested the effect of adding Jac1 and the possible substrate proteins simultaneously. The ATPase activity of Ssq1 was stimulated 4.9- or 2.8-fold when Jac1 and Isu1 or IscU, respectively, were both added at a concentration equimolar to that of Ssq1. When Isu1 or IscU was present at a concentration 5-fold higher than Ssq1 and Jac1, 10.4- and 8.1-fold stimulation, respectively, was observed (Fig. 1*C*). Moreover, we also ob-

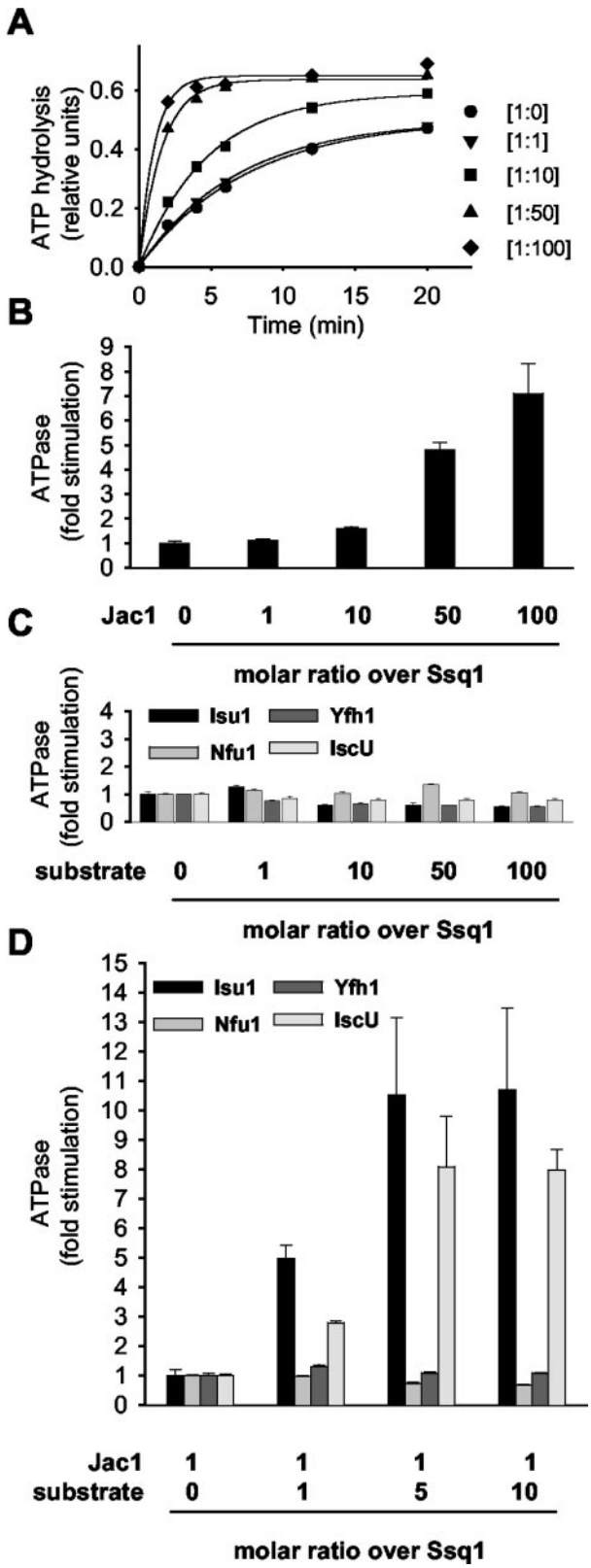

FIG. 1. **Effects of Jac1 and potential substrates on the single turnover ATP hydrolysis by Ssq1·ATP complex.** *A*, the Ssq1·[α-^{32}P]ATP complex (0.2 μM) was incubated at 25 °C with different concentrations of purified Jac1, indicated as a molar ratio of [Ssq1:Jac1]. *B*, rates of ATP hydrolysis calculated from *A*, presented as -fold of stimulation. The ATPase rate of Ssq1 protein alone was set as 1. *C*, the Ssq1·[α-^{32}P]ATP complex (0.2 μM) was incubated with different concentrations of purified proteins: Isu1, Yfh1, Nfu1, and IscU as described in *A*, and the -fold ATPase stimulation was calculated as described in *B*. *D*, the Ssq1·[α-^{32}P]ATP complex (0.2 μM) was incubated with Jac1 (0.2 μM) and different concentrations of Isu1, Yfh1, Nfu1, and IscU as indicated.

served, that even at substoichiometric concentrations (5-fold lower than Ssq1) Jac1 was able to stimulate Ssq1 ATPase providing that Isu1 was present in the reaction mixture (data not shown). However, no enhancement of stimulation was observed in the presence of either Nfu1 or Yfh1, regardless of protein concentration. From these results we concluded that Isu1 behaves as a native protein substrate of Ssq1, and that Jac1 cooperates with it in stimulating ATPase activity.

Jac1 Binds to Isu1 and Stimulates Ssq1-Isu1 Interaction—It was previously shown that Hsc20 binds directly to IscU (17). Therefore, we tested whether Jac1 is able to interact with Isu1. Using purified SPR for this purpose, Jac1 was passed over the surface of a sensor chip to which purified Isu1 was immobilized by random cross-linking. The increase of refractive index (expressed in arbitrary response units) was proportional to the concentration of Jac1 passed over the chip surface, indicating a specific interaction between these two proteins (Fig. 2*A*).

The ability of Jac1 to interact directly with Isu1 and the ability of Jac1 and Isu1 to cooperatively stimulate the ATPase activity of Ssq1, suggested that Jac1 might target the substrate Isu1 to Ssq1. To begin to test this idea, we monitored the effect of flowing both Jac1 and Ssq1 over Isu1 bound to the sensor chip. Because it is clear that physiologically important, rapid association of Hsp70 with substrates occurs in the ATP state, we first tested the interaction between Ssq1 and Isu1 in the presence of ATP. When Ssq1 alone at 0.8 μM concentration was passed over the chip surface, only a weak signal (20 response units) was detected, indicating that in the presence of ATP Ssq1 was not able to strongly interact with Isu1 (Fig. 2*B*). Next, we tested if the presence of Jac1 resulted in enhanced binding to Isu1. A mixture containing 0.7 μM Jac1, a concentration at which alone shows a response of only 40 response units (Fig. 2*A*), and 0.8 μM Ssq1 was passed over the Isu1 surface. An enhanced signal of 160 response units was observed. We interpreted this enhanced signal as evidence for stimulation of Ssq1-Isu1 interaction by Jac1.

Based on general principles of Hsp70 and J-protein action, the enhancement of the SPR signal in the presence of both Ssq1 and Jac1 is consistent with Jac1 binding to Isu1 first and then promoting its interaction with Ssq1. To more directly test this idea, we tested interactions among these three proteins in solution by centrifugation of 5 μM solutions of these proteins through glycerol gradients. Upon centrifugation of either Jac1 or Isu1 alone, the peak of Isu1 was found in fractions 4–7, and that of Jac1 in fractions 6–9 (Fig. 3, *A* and *B*). However, when a mixture of these two proteins was subjected to centrifugation, a portion of both proteins co-localized to fractions 7–10 (Fig. 3*C*). Quantitative analysis indicated that at least 40% of Isu1 protein co-localized with Jac1. This movement of both proteins toward the bottom of the test tube was interpreted as formation of an Isu1·Jac1 complex.

When Isu1 was incubated with Ssq1 in the presence of ADP, about 23% of Isu1 co-migrated with Ssq1 in fractions 13–17, indicating that Isu1 and Ssq1 could interact (Fig. 3*D*). In contrast, we did not detect complex formation between Ssq1 and Isu1 in the presence of ATP, as both proteins migrated during glycerol gradient centrifugation to the positions characteristic for individual proteins in the control experiment (Fig. 3*E*). However, when Jac1 was present at the same concentration as Ssq1 and Isu1 (5 μM each) in the presence of ATP, two separate protein complexes were detected following glycerol gradient centrifugation. In fractions 6–10, 46% of Isu1 co-migrated with Jac1 and in fractions 12–17, 51% of Isu1 co-migrated with Ssq1 (Fig. 3*F*), indicating substrate binding by Hsp70 chaperone. Interestingly, only minute amounts of Jac1 were visible in fractions corresponding to the Ssq1·Isu1 complex. From these experiments we concluded that Jac1 was able to bind substrate, Isu1, and subsequently target it to Ssq1, but did not remain associated with the Ssq1·Isu1 complex.

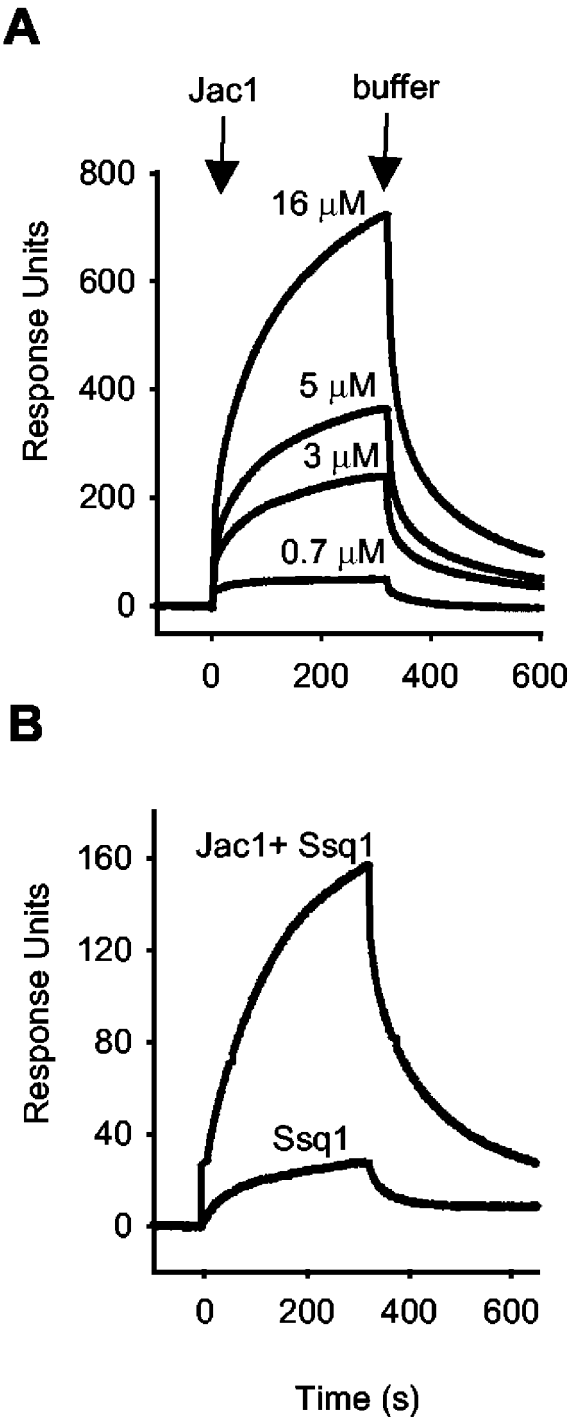

FIG. 2. **SPR analysis of interactions among Jac, Isu1, and Ssq1 in the presence of ATP.** Purified Isu1 protein was randomly cross-linked to the surface of a SPR chip (2000 response units). *A*, at the zero time point an equilibration solution (buffer R and 2 mM ATP) passing over the chip was replaced by a solution containing Jac1 at the indicated concentrations. At 300 s the solutions were changed back to equilibration buffer. *B*, purified Ssq1 protein (0.7 μM), or a mixture of Ssq1 (0.7 μM) and Jac1 (0.8 μM) were passed over the chip with immobilized Isu1 as described in *A*.

Ssq1 Differs from Hsc66 in Stability of ATP Binding—Cycles of substrate binding and release are essential for the biological functions of Hsp70 chaperones. The rate of release of a substrate protein is determined by the rate of release of ADP.

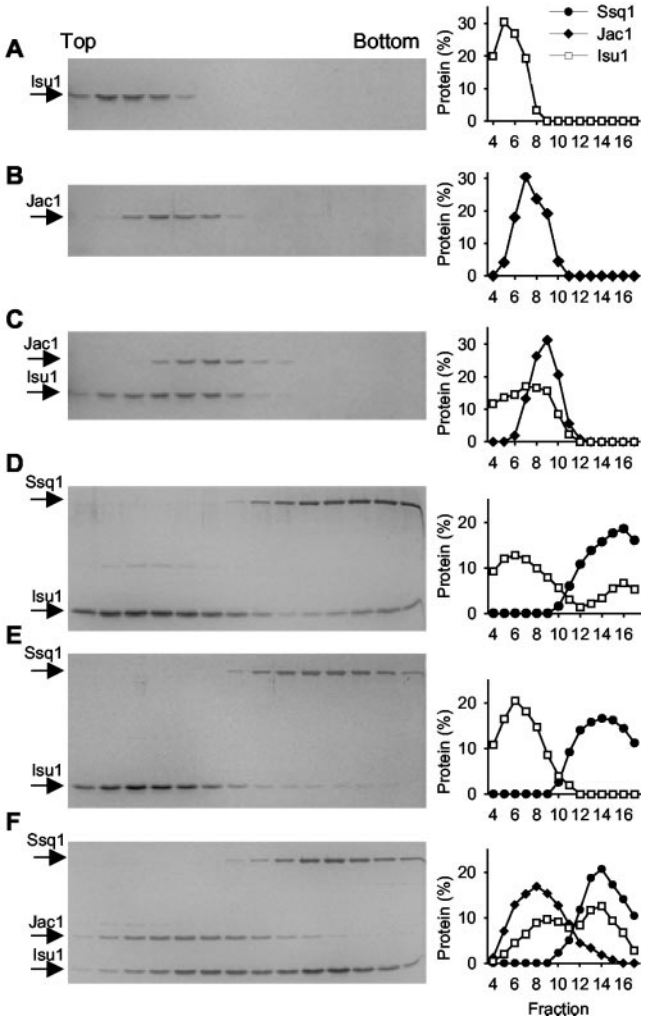

FIG. 3. **Jac1 binds Isu1 and activates Ssq1 for Isu1 binding in the presence of ATP.** The sedimentation of Isu1 (*A*), Jac1 (*B*), Isu1 and Jac (*C*), Isu1 and Ssq1 in the presence of ADP (*D*), Isu1 and Ssq1 in the presence of ATP (*E*), Isu1, Jac1, and Ssq1 in the presence of ATP (*F*) in 3 ml of 15–35% (v/v) glycerol gradient in buffer B. Each protein was present in the reaction mixture (70 μl) at 5 μM concentration. ADP or ATP, as indicated, was present in both the reaction mixture and in the glycerol gradient at 2 mM concentration. Fractions were collected from the top of the gradient and their protein contents were assessed by SDS-PAGE followed by silver staining. Plots representing quantification of protein content were obtained by densitometry analysis using Quantity One software (Bio-Rad).

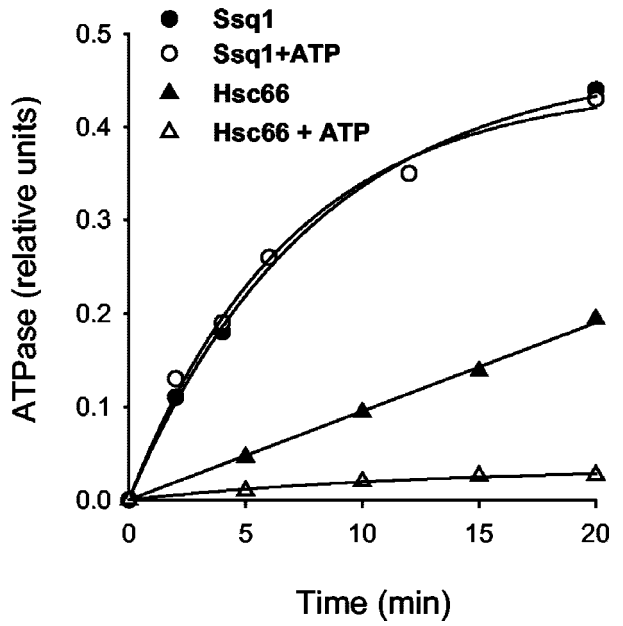

FIG. 4. **Different effects of ATP excess on single turnover ATP hydrolysis by HscA·ATP and Ssq1·ATP complexes.** Kinetics of ATP hydrolysis by HscA·[α-^{32}P]ATP and Ssq1·[α-^{32}P]ATP complexes were measured as described in the legend to Fig. 1, in the absence and presence of 100 μM unlabeled ATP.

Whether a co-chaperone is needed to facilitate nucleotide release depends on intrinsic properties of the Hsp70. We decided to compare the stability of nucleotide binding of Ssq1 with that of Hsc66, which has been shown to have a rapid nucleotide off-rate. We compared the rates of ATP hydrolysis by preformed radioactive Hsc66·[α-^{32}P]ATP and Ssq1·[α-^{32}P]ATP complexes in the absence and presence of excess unlabeled ATP (Fig. 4). As expected, the presence of unlabeled ATP strongly inhibited ATP hydrolysis by the Hsc66·[α-^{32}P]ATP complex, because the unlabeled ATP replaced the radioactive nucleotide bound to Hsc66 prior to its hydrolysis (Fig. 4). In contrast, the excess of unlabeled ATP had no effect on ATP hydrolysis by Ssq1·[α-^{32}P]ATP complex, suggesting that the Ssq1-ATP interaction must be relatively stable. This result indicates a significant biochemical difference between mitochondrial and bacterial Hsp70s involved in biogenesis of Fe/S centers.

Mge1 Is a Nucleotide Release Factor for Ssq1—Because of the stable interaction between Ssq1 and ATP, we predicted that Ssq1 might require a nucleotide release factor for stimulating the nucleotide exchange during the protein substrate binding cycle. Because the only known nucleotide release factor in mitochondria is Mge1, co-chaperone of the major mitochondrial Hsp70, Ssc1, we tested the ability of Mge1 to directly interact with Ssq1 using SPR. Purified Mge1 was immobilized on the sensor chip by random cross-linking, then purified Ssq1 was passed over the surface of the chip. In the absence of nucleotide, a strong increase of response units indicated direct interaction between these two proteins. In contrast, no binding was detected in the presence of ATP (Fig. 5*A*). This nucleotide sensitivity of binding was previously observed for the interaction of Mge1 with Ssc1 (35), as well as for the interaction of the homologous bacterial chaperones GrpE and DnaK (36), and was thus interpreted as an indication of a specific and functional interaction between these chaperones.

To test the effect of Mge1 on the release of bound nucleotide from Ssq1, isolated Ssq1·[α-^{32}P]ATP complex was incubated in the presence of Mge1. The reaction mixture was again subjected to gel filtration chromatography to monitor the release of bound nucleotide. Although a significant amount of radioactivity (49%) remained associated with Ssq1 in the absence of Mge1, a peak of free nucleotide was also observed (Fig. 5*B*). Moreover, the presence of excess unlabeled ATP did not affect the amount of radioactivity associated with Ssq1 (47%), indicating again that the association between Ssq1 and nucleotide was very stable. Analysis of the peak fractions revealed that over 90% of nucleotide bound to Ssq1 was ATP, whereas the free nucleotide peak contained mostly (80–90%) ADP (Fig. 5*C*). This distribution of nucleotides suggested that ADP was preferentially released from Ssq1, and ATP was relatively stably bound. This stability of bound ATP was consistent with the results shown on Fig. 4, as excess of unlabeled ATP did not quench hydrolysis of ATP by pre-formed Ssq1·ATP complex.

In contrast, when Mge1 was incubated with the Ssq1·[α-^{32}P]ATP complex the peak of radioactivity associated with Ssq1 decreased significantly (to 10%) and almost completely disappeared when Mge1 and unlabeled ATP were present concomitantly. Moreover, in the presence of Mge1, the peak of free nucleotide contained significantly more ATP (55% of total) (Fig.

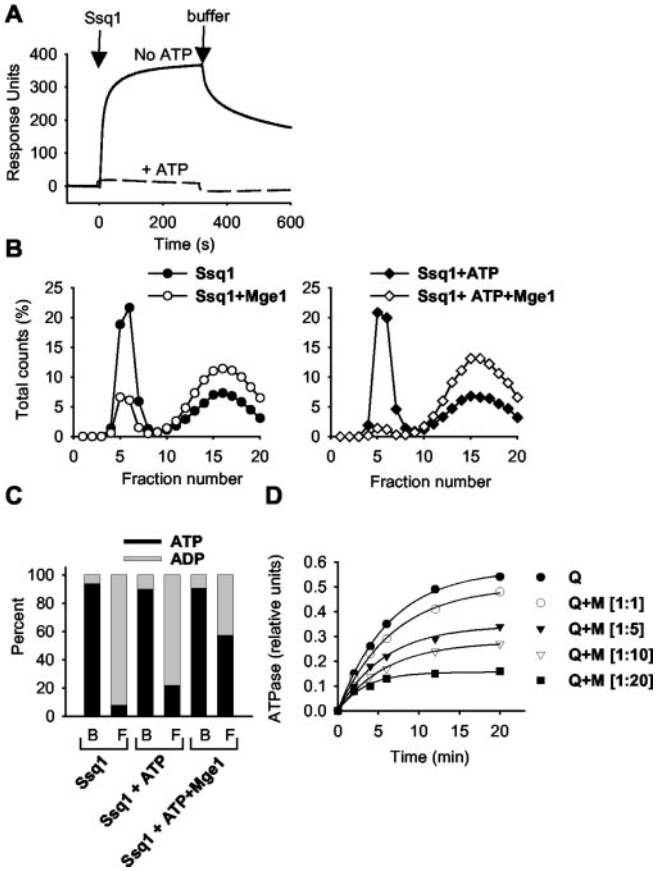

FIG. 5. **Mge1 is a nucleotide release factor for Ssq1 protein.** *A*, Mge1-Ssq1 interaction. Purified Mge1 was randomly cross-linked to the surface of a SPR chip (1000 response units). Purified Ssq1 (0.7 μM) was injected into solution passing over the chip in the absence of nucleotide or in the presence of ATP (2 mM). *B*, Mge1 releases both ATP and ADP from Ssq1. The Ssq1·[α-^{32}P]ATP complex (0.93 μM) was incubated at 25 °C for 10 min in the absence or presence of 100 μM unlabeled ATP to achieve 50–60% conversion of ATP to ADP, then Mge1 at 9.3 μM was added, where indicated, to the reaction mixture, which was loaded onto a gel filtration column. Fractions were collected and aliquots were counted. Percentage of total counts in each fraction was plotted. The first peak of radioactivity, fractions 5 to 6, corresponds to bound nucleotides, whereas the second peak, from fractions 12 to 20 corresponds to free nucleotides. *C*, relative amounts of ATP and ADP in bound (*B*) and free (*F*) pooled fractions of nucleotides. Aliquots of each fraction were analyzed by thin-layer chromatography on PEI-cellulose, followed by PhosphorImager quantification of ATP and ADP. *D*, Mge1 inhibits single turnover ATP hydrolysis by Ssq1·ATP complex. Kinetics of ATP hydrolysis by Ssq1·[α-^{32}P]ATP complex (0.2 μM) was measured in the presence of Mge1 at the indicated molar ratios [Ssq1:Mge1].

5*C*), supporting the idea that Mge1 was responsible for release of both ATP and ADP from Ssq1, and thus, functioned as a nucleotide release factor. Consistent with that conclusion, hydrolysis of [α-^{32}P]ATP bound to Ssq1 was inhibited in the presence of Mge1 (Fig. 5*D*), whereas kinetic analysis indicated that the initial rates of ATP hydrolysis were the same regardless of the amount of Mge1 present in the reaction mixture (data not shown). The latter result is consistent with the function of Mge1 as a nucleotide release factor, not an inhibitor of the hydrolysis of ATP by Ssq1; because, in the presence of both Mge1 and an excess of unlabeled ATP, the radioactive nucleotide from the Ssq1·ATP complex was released before it was hydrolyzed.

Activation of Ssq1 ATPase under Steady-state Conditions Depends on the Presence of Jac1, Mge1, and Isu1—Because Mge1 is a nucleotide release factor for Ssq1, we asked whether the presence of this protein affected Ssq1 ATPase activity measured under steady-state conditions. Under such conditions, the overall rate of ADP formation depends on the rates of the following three stages of the reaction: ATP binding, ATP hydrolysis, and dissociation of the reaction products, ADP and inorganic phosphate. The steady-state ATPase was measured at 25 °C, for 0.8 μM Ssq1 at saturating (118 μM) concentration of ATP. The k_{cat} calculated from this experiment was 0.0047 ± 0.0003 s^{-1}. This value is about 3.4-fold higher than the k_{cat} published for Hsc66 (20).

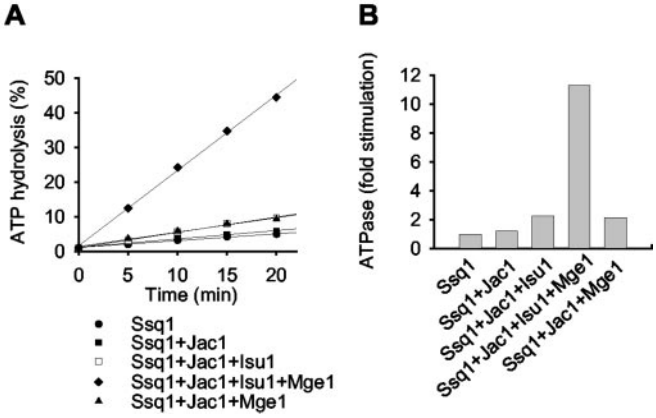

FIG. 6. **Efficient stimulation of steady-state ATPase of Ssq1 requires the presence of Mge1.** *A*, kinetics of steady-state ATP hydrolysis. The 80-μl incubation mixtures contained 0.8 μM Ssq1, 0.8 μM Jac1, 0.8 μM Isu1, 0.8 μM Mge1. The concentration of ATP was 118 μM. Experimental details are described under "Experimental Procedures." *B*, fold stimulation of ATPase hydrolysis. Data from *A* was quantified; the ATPase rate of Ssq1 alone was set as 1.

When an equimolar amount of Jac1 was added to the reaction mixture the ATPase rate did not increase over that found in the absence of Jac1 (Fig. 6), consistent with the weak effect of low Jac1 concentrations on Ssq1 ATPase measured under single turnover conditions (Fig. 1, *A* and *B*). However, only a 2-fold increase of ATPase activity was observed in the presence of both Jac1 and Isu1, whereas these two proteins together stimulated 4.9-fold under single turnover conditions (Fig. 1*D*). But, when Mge1 was added to the reaction mixture containing Jac1 and Isu1, Ssq1 ATPase was stimulated 11-fold. Thus, the weak stimulation of ATPase by Jac1 and Isu1 under steady-state conditions, which is strongly enhanced by Mge1, indicates that the release of ADP is the rate-limiting step and thus, a nucleotide release factor is required for efficient stimulation. Interestingly, in the presence of co-chaperones (Jac1 and Mge1), but without the protein substrate Isu1, 2-fold stimulation of Ssq1 ATPase was observed. The latter observation once more points to a very specific interaction between Ssq1 and its protein substrate Isu1, as a prerequisite for functional stimulation of ATPase activity.

To further characterize interactions between Ssq1 and its co-chaperones and substrate, we measured the rate of ATPase hydrolysis at different concentrations of individual proteins (Fig. 7). These results revealed that maximal stimulation (12-fold) of Ssq1 ATPase occurred when Jac1 was present at a 2:1 molar ratio over Ssq1. Similar optimal molar ratios were found for Isu1 and Mge1. Moreover, at concentrations higher than optimal, both Jac1 and Isu1 had very little effect on ATP hydrolysis rate. In contrast, concentrations of Mge1 higher than optimal resulted in inhibition of Ssq1 ATPase activity; for example, at a 10:1 molar ratio, hydrolysis was inhibited by 40%.

Mdj1 Cannot Functionally Replace Jac1—Our finding that Mge1 functions as a nucleotide release factor for Ssq1 suggests that two Hsp70 chaperones present in mitochondria, Ssq1 and Ssc1, might share at least one co-chaperone protein. This result raised the question as to whether the J-protein partner of Ssc1,

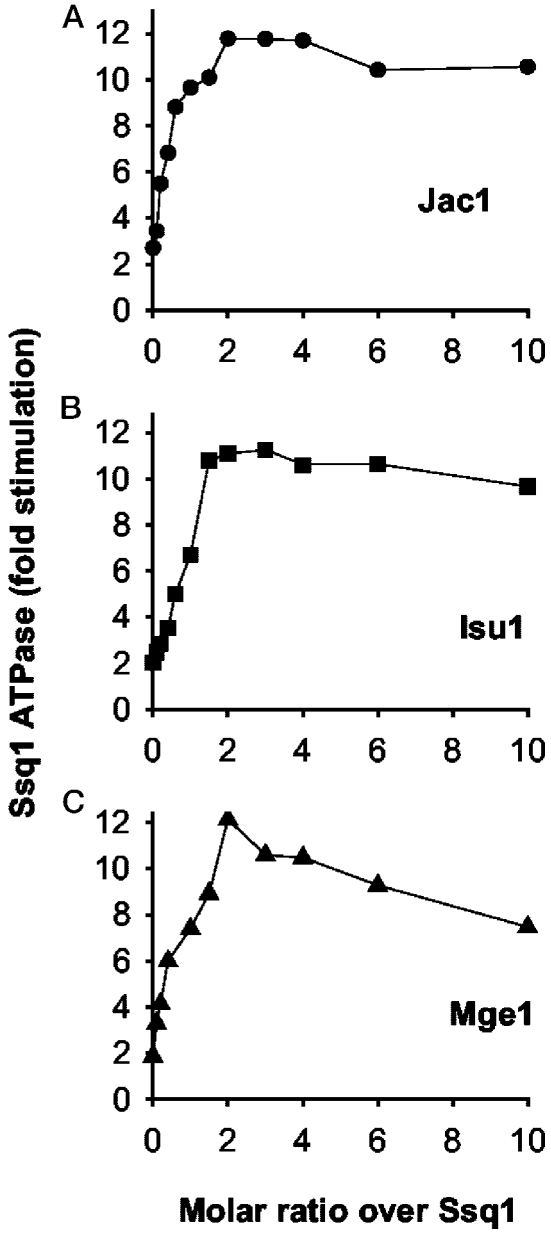

FIG. 7. **Titration of Jac1, Isu1, and Mge1 requirements.** *A*, reaction mixture of Ssq1 (0.8 μM), Isu1 (0.16 μM), and Mge1 (0.8 μM) was incubated with various amounts of Jac1. *B*, Ssq1 (0.8 μM), Jac1 (0.8 μM), and Mge1 (0.8 μM) were incubated with various amounts of Isu1. *C*, Ssq1 (0.8 μM), Jac1 (0.8 μM), and Isu1 (0.16 μM) were incubated with various amounts of Mge1. ATP was at a concentration of 118 μM in all assays.

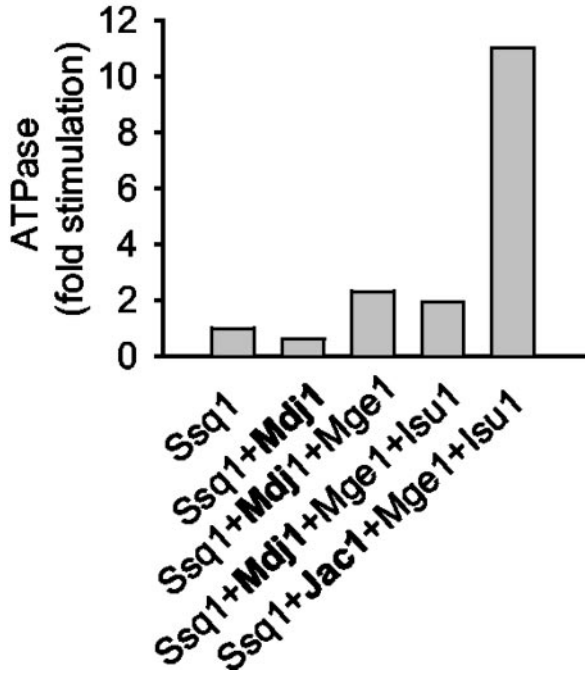

FIG. 8. **Mdj1 cannot replace Jac1 in stimulation of steady-state ATPase of Ssq1.** Kinetics of steady-state ATP hydrolysis by Ssq1 was measured in the presence of the indicated proteins as described in the legend to Fig. 6. Mdj1 was present at 0.8 μM and ATP at 118 μM.

Mdj1, is also shared with Ssq1. Thus, we substituted Mdj1 for Jac1 in the assays described above. Unlike Jac1, Mdj1 did not stimulate Ssq1 ATPase activity under either steady-state conditions in the presence of Isu1 and Mge1 (Fig. 8) or under single turnover conditions in the presence of Isu1 (data not shown). Moreover, Mdj1 could not enhance the interaction of Ssq1 with Isu1 as assessed by glycerol gradient centrifugation (data not shown).

DISCUSSION

Interaction of Ssq1 and Jac1 with the Substrate Protein Isu1—The results reported here indicate that the mitochondrial chaperones Ssq1 and Jac1 can both bind the native substrate protein Isu1, the presumptive scaffold for formation of Fe/S centers (34). More importantly, they act as partners, which is consistent with a model in which Isu1 is targeted to Ssq1 in a Jac1-dependent manner. Ssq1/ATP, does not interact efficiently with Isu1 protein alone, as shown by both SPR and glycerol gradient centrifugation experiments. But in the presence of Jac1, a stable Ssq1·Isu1 complex formed, even though no Jac1·Ssq1 or Jac1·Ssq1·Isu1 complexes were detected. In addition, both Isu1 and Jac1 are required for efficient stimulation of Ssq1 ATPase activity. These results are consistent with a model in which Jac1 targets Isu1 to Ssq1, but does not remain part of the complex.

The best studied example of a J-protein-dependent substrate targeting is that of DnaJ-dependent targeting of bacterial σ32 protein for binding by DnaK. In that case, DnaJ is required for interaction of DnaK in the presence of ATP and σ32 (18) and DnaJ must be present simultaneously for efficient stimulation of the ATPase activity of DnaK (37). Based on these similarities between the DnaK/DnaJ/σ32 and the Ssq1/Jac1/Isu1 systems and what is generally known about J-protein-Hsp70 interactions (16), one can hypothesize that the Jac1·Isu1 complex interacts simultaneously with two distinct functional domains of Ssq1: the J-domain of Jac1 with the N-terminal ATPase domain of Ssq1, and Isu1 with the C-terminal substrate binding domain of Ssq1. According to this model, one would expect the conserved HPD motif of the J-domain (38) to be essential for Jac1 activity, as the J-domain of other J-proteins has been shown to be required for interaction with the ATPase domain of the Hsp70 chaperone resulting in ATPase stimulation and subsequent conformational changes resulting in stabilization of substrate interaction (16). Indeed, alteration of the Jac1 HPD motif strongly compromised Jac1 *in vivo* function (4). Thus, it is likely that the "true substrate" for Ssq1 is not free Isu1, but rather, Isu1 in complex with Jac1. But because no significant amount of Jac1 is present in the Ssq1·Jac1 complex, and because Jac1 was able to stimulate the ATPase activity at substoichiometric concentrations in relation to Isu1 and Ssq1, we conclude that Jac1 acts catalytically.

How specific is the interaction of Ssq1 and Jac1 with Isu1? According to the model presented above, substrate specificity of Ssq1 is determined by two important interactions: Jac1 binding

to the potential substrate and recognition of Jac1-substrate complex by Ssq1. We found that neither Nfu1 nor Yfh1 proteins were able to stimulate the Ssq1 ATPase activity in the presence of Jac1, nor did they interact with Jac1 (data not shown). Thus, two other mitochondrial proteins known to be involved in Fe/S biogenesis are not implicated as Jac1/Ssq1 substrate proteins. Negative results regarding the interaction of Yfh1 and Ssq1 obtained during this study are particularly interesting because results from *in vitro* experiments indicate that Ssq1 must be present for efficient processing at one step of the import process (7, 39). The lack of interaction with the mature protein indicates that while the precursor or partially folded form might interact *in vivo*, the mature folded protein does not appear to be a substrate of Ssq1 like the Isu1 protein.

In summary, the interactions of Ssq1/Jac1 with the specific protein substrate Isu1 are strikingly consistent with interactions, discussed in the Introduction, found among their bacterial counterparts, Hsc66/Hsc20 and IscU substrate protein. Not only do both the J-protein and Hsp70 bind closely related substrate proteins, stimulation of the ATPase activity of Hsp70 is significantly enhanced only when both the J-protein and the substrate protein are present (17). Moreover, in bacteria, a stable complex between Hsc20-IscU and Hsc66-IscU has been detected *in vivo* (40). In addition, bacterial IscU stimulated the ATPase activity of Ssq1 in the presence of Jac1, and was able to form a stable complex with Jac1. Thus, at this point in time, data from both bacteria and mitochondria indicate that chaperones involved in biogenesis of Fe/S centers are highly conserved with respect to their protein substrate recognition, and thus, likely in their biological function as well. Because Isu/IscU are scaffolds for the assembly of Fe/S centers to be transferred to other proteins, the chaperones could assist in conformational changes required for either assembly of the Fe/S on the scaffold, or transfer to a recipient protein. Distinguishing between these possibilities will require rigorous testing.

The Interaction of Ssq1 with Nucleotide and the Nucleotide Release Factor Mge1—Besides the similarities described above, a major biochemical difference between prokaryotic and eukaryotic chaperone systems involved in Fe/S centers biogenesis was also observed. In bacteria, the Hsc66/Hsc20 system appears to be functionally isolated from the Hsp70 machinery consisting of DnaK, DnaJ, and GrpE proteins. DnaJ is unable to stimulate the ATPase activity of Hsc66 (13). Similarly in the mitochondrial system, Mdj1 does not stimulate ATP hydrolysis by Ssq1.

However, the situation is more complicated in the case of nucleotide release factors. Because of the low affinity of Hsc66 for nucleotide, it is thought that no factor is needed to aid the release of the products of ATP hydrolysis. Indeed, whereas DnaK requires GrpE, no interaction of Hsc66 with GrpE has been observed (13, 21). Like DnaK, Ssc1, the abundant Hsp70 of the mitochondrial matrix (31), has a high affinity for adenine nucleotides. Thus, similar to DnaK, Ssc1 would be predicted to require a nucleotide release factor to enhance dissociation of ATP hydrolysis products, and hence, to stimulate cycles of binding and releasing of protein substrates. Mge1, the orthologue of bacterial GrpE, has been shown to bind Ssc1 in an ATP-sensitive manner and stimulate the release of both ADP and ATP from Ssc1 (31, 35, 42).

The biochemical results presented here demonstrate that Ssq1 has very similar properties to those of Ssc1 in regards to interaction with nucleotide. Thus, *in vivo*, a requirement for a nucleotide release factor for Ssq1 would be expected. Because Mge1 can stimulate the release of nucleotide from Ssq1, the hypothesis that Mge1 functionally interacts with Ssq1 *in vivo*, thus playing an important role in Fe/S center biogenesis has emerged. But, although these biochemical results, as well as published *in organellar* studies (5, 43), indicate that Mge1 can interact with Ssq1 as a nucleotide release factor, it has not been unequivocally established that in the living cell Mge1 plays an essential role in biogenesis of Fe/S centers. Because Mge1 is required for the function of the essential Hsp70 Ssc1, the interpretation of analyses of *MGE1* mutants to address this question presents significant challenges. It remains possible that a yet to be identified factor plays the role of a nucleotide release factor for Ssq1, and thus Fe/S center biogenesis.

The presence of Hsp70 proteins specialized in Fe/S biogenesis both in bacteria and mitochondria might seem to easily lead to the suggestion that the mitochondrial counterpart evolved directly from its bacterial ancestor. Therefore, it was surprising to find differences in nucleotide binding and interactions with nucleotide release factor. However, a recently published phylogenetic analysis in which the nucleotide sequences of Hsp70 genes derived from 56 completely sequenced prokaryotic and eukaryotic genomes were compared indicates that the mitochondrial Hsp70 Ssq1 is more closely related to Ssc1 than to bacterial Hsc66 (41). Therefore, it was proposed that during evolution of mitochondria the bacterial gene encoding Hsc66 was replaced by its paralog *SSQ1*. Thus, it is possible that biochemical similarities between Ssq1 and Ssc1 reflect the common origin of these proteins. One can imagine that the ability to modulate the rate of nucleotide release, and thus the rate of chaperone cycling could be advantageous to the cell, perhaps serving as a regulatory mechanism to couple protein folding and Fe/S center insertion. On the other hand, one should seriously consider a null hypothesis that the Mge1-Ssq1 interaction may only reflect structural similarities related to the common origin of the *SSQ1* and *SSC1* genes.

Conclusions—The results presented here demonstrate that a critical protein in Fe/S formation, Isu1, is a substrate for the mitochondrial chaperones Ssq1 and Jac1, and that these chaperones function as partners to facilitate binding of Isu1 to Ssq1. These conclusions are consistent with previous genetic and cell biological data indicating an *in vivo* role for these chaperones in Fe/S center formation. Therefore, the Ssq1/Jac1 system is a suitable model system for future analysis of the role of molecular chaperones in eukaryotic Fe/S center assembly using biochemical, as well as *in vivo* approaches.

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