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The Hsp70 Chaperone Ssq1p Is Dispensable for Iron-Sulfur Cluster Formation on the Scaffold Protein Isu1p*[S]

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The specialized yeast mitochondrial chaperone system, composed of the Hsp70 Ssq1p, its co-chaperone J-protein Jac1p, and the nucleotide release factor Mge1p, perform a critical function in the biogenesis of iron-sulfur (Fe/S) proteins. Using a spectroscopic assay, we have analyzed the potential role of the chaperones in Fe/S cluster assembly on the scaffold protein Isu1p *in vitro* in the presence of the cysteine desulfurase Nfs1p. In the absence of chaperones, the kinetics of Fe/S cluster formation on Isu1p were compatible with a chemical reconstitution pathway with Nfs1p functioning as a sulfide donor. Addition of Ssq1p improved the rates of Fe/S cluster assembly 3-fold. However, this stimulatory effect of Ssq1p required neither ATP nor Jac1p and could be fully attributed to the activation of the Nfs1p desulfurase activity by Ssq1p. Furthermore, chaperone-stimulated Fe/S cluster assembly did not involve the specific interaction between Isu1p and Ssq1p, since the effect was observed with Isu1p mutant proteins defective in this interaction, suggesting that nonspecific binding of Ssq1p to Nfs1p helped to prevent its unfolding. Consistent with this idea, these Isu1p mutants were capable of binding an Fe/S cluster *in vivo* but failed to restore the growth and Fe/S cluster assembly defects of a Isu1p/Isu2p-deficient yeast strain. Taken together, these data suggest that Ssq1p/Jac1p/Mge1p are not important for Fe/S cluster synthesis on Isu1p. Hence, consistent with previous *in vivo* data, these chaperones likely function in steps subsequent to the *de novo* synthesis of the Fe/S cluster on Isu1p.

Proteins with iron-sulfur (Fe/S) cofactors play important roles in fundamental cellular processes such as redox reactions, metabolic catalysis, and regulation of gene expression (1–3). In eukaryotes such as *Saccharomyces cerevisiae*, mitochondria perform a central function in the biosynthesis of cellular Fe/S proteins (4). The mitochondrial iron-sulfur cluster (ISC)[4] assembly machinery is essential for the biosynthesis of mitochondrial Fe/S proteins and is also required for the maturation of cytosolic and nuclear Fe/S proteins (5). The ISC system likely has been inherited from bacteria that encode proteins of similar structure and function in the *isc* operon that is widely distributed throughout the bacterial kingdom (6, 7).

Fe/S cluster assembly in yeast mitochondria is initiated by the release of sulfur from cysteine catalyzed by the cysteine desulfurase Nfs1p (8–10). The highly conserved proteins Isu1p and Isu2p play a critical role in biogenesis by providing a scaffold for transient Fe/S cluster formation. The protein pair Isu1p/Isu2p is essential for cell viability, and loss of function causes severe defects in cellular Fe/S protein maturation (11–13). Purified Isu1p and its bacterial homologs IscU/NifU bind mononuclear iron and Fe/S clusters that can be assembled by chemical or desulfurase-directed reconstitution (3, 7, 14). Three conserved cysteine residues of the Isu proteins are essential for the binding of iron or Fe/S clusters. These residues are located on the tip of the molecule and are exposed to the solvent (15). The preformed Fe/S cluster can be transferred from Isu1p/IscU to a recipient Fe/S apoprotein *in vitro*, indicating that this class of proteins serves as a scaffold for the *de novo* synthesis of Fe/S clusters before their transfer and integration into apoproteins (14, 16–18). Fe/S cluster formation on yeast Isu1p has been demonstrated *in vivo* requiring the function of Nfs1p, the ferredoxin Yah1p and Yfh1p (19, 20).

In yeast mitochondria, a specialized Hsp70 chaperone, Ssq1p, plays a critical role in Fe/S protein biogenesis. Ssq1p co-operates with its cognate J-type co-chaperone Jac1p and the nucleotide exchange factor Mge1p, and reduction in the levels of components of this machinery is associated with severe growth defects and an impairment of cellular Fe/S protein maturation (21–24). Ssq1p binds to Isu1p/Isu2p with high specificity by interacting with a highly conserved PVK tripeptide motif on the Isu proteins (25, 26). Alterations in this motif disrupt the interaction with Ssq1p and result in deleterious *in vivo* phenotypes (27). Conversely, *SSQ1* mutant proteins with severely reduced affinities for the Isu proteins display a similar phenotype as *SSQ1* deletion cells, underscoring the importance of the Ssq1p-Isu protein interaction *in vivo* (28). This interaction seems to be conserved for all Hsp70s specialized for the biogenesis of Fe/S proteins, including those in bacteria (25, 29, 30).

The cycle of interaction of Ssq1p with Isu1p is similar of that of any Hsp70 with client proteins. Under physiological conditions, the Isu proteins encounter the Hsp70 in its ATP-bound state, and binding is stabilized upon hydrolysis of ATP. The cycle of interaction is completed when ADP is exchanged for ATP, and the Isu protein substrate is released (23, 31, 32). The Ssq1p ATPase activity is stimulated by interaction with the co-chaperone Jac1p which in addition actively promotes complex formation between Ssq1p and Isu1p by specifically binding to Isu1p (24, 26, 33). Mutations within the J-domain of Jac1p result in decreased activity *in vitro* and cause profound growth defects *in vivo*

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[4] The abbreviation used is: ISC, iron-sulfur cluster assembly.

(22, 33). Thus ATP binding and hydrolysis stimulated by a J-protein are required for function as expected of any Hsp70 machinery *in vivo*. However, the precise mechanistic role of this chaperone system in Fe/S cluster biogenesis is currently unresolved. In yeast cells, depletion of both Ssq1p and Jac1p causes an accumulation of Fe/S clusters on Isu1p suggesting that these chaperones are likely required for steps following the *de novo* synthesis of the Fe/S clusters on the Isu scaffold proteins (20).

To critically test the role of chaperones, we have initiated experiments to reconstitute Fe/S protein assembly using components of *S. cerevisiae*. In this communication, we report on *in vitro* and *in vivo* analyses on the possible role of Ssq1p, Jac1p, and Mge1p in the *de novo* formation of Fe/S clusters on Isu1p. We developed an experimental *in vitro* system that allowed the time-resolved detection of Fe/S cluster formation on Isu1p. We provide evidence that Ssq1p and Jac1p do not specifically promote the rates of *de novo* Fe/S cluster formation on Isu1p. These *in vitro* conclusions were supported by investigations *in vivo* using Isu1p mutants impaired in the interaction with Ssq1p. Thus, our work supports the conclusion that the Hsp70 chaperones specialized in the biogenesis of Fe/S proteins are likely required after Fe/S cluster synthesis on Isu1p, *e.g.* during transfer of the Fe/S cluster to recipient apoproteins.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—The following strains of *S. cerevisiae* were used: W303-1A (*MATa, ura3-1, ade2-1, trp1-1, his3-11,15, leu2-3,112*) served as wild type; Gal-ISU1/Δisu2 (34), Gal-NFS1, and Gal-SSQ1 (20). Cells were grown in rich yeast extract/peptone, synthetic complete minimal or "iron-poor" minimal (lacking added iron chloride) medium, containing the required carbon sources (35). For overproduction of Isu1p, wild-type *ISU1* and the mutant alleles with the P134S, V135E, and K136A amino acid exchanges (27) were inserted into yeast vector p426-GPD under the control of the strong *TDH3* promoter (36).

Recombinant Proteins—Nfs1p from *S. cerevisiae* was purified from *Escherichia coli* strain HMS174(DE3) that co-expressed *NFS1* from plasmid pET15b-NFS1 and *E. coli groEL* from pOFX*tac*SL2 using hydrophobic interaction and gel filtration chromatography as described previously (10). Recombinant, His-tagged versions of Jac1p (22), Ssc1p (12, 37), Mge1p and Mdj1 (33, 38, 39), and Ssq1p and Isu1p were purified as described (24). These recombinant proteins were functional, as the corresponding yeast deletion strains were rescued by low copy plasmids containing the corresponding coding sequences. Polysulfane modifications associated with purified Isu1p were removed by treatment with 100 mM KCN for 1 h at 25 °C. KCN was subsequently removed by gel filtration.

In Vitro Fe/S Reconstitution Assays—Fe/S cluster reconstitution experiments were carried out under anaerobic conditions essentially as described previously (40). A standard reaction mixture containing Nfs1p (0.8 μM final concentration) in 220 μl of buffer A (20 mM Tris, pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 10 μM pyridoxal phosphate, 0.2 M sucrose, 5 mM dithiothreitol, 4 mM cysteine, 0.3 mM ferric ammonium citrate) was incubated at 25 °C for 20 min in an anaerobic chamber (Coy Instruments). When necessary Ssq1p, Jac1p, and Mge1p supplemented with 0.25 mM ATP were added to the reaction mixtures at the same time as Nfs1p. Isu1p was added, and the mixture was transferred under anaerobic conditions to a quartz cuvette, sealed tightly, and inserted into a double beam spectrophotometer (model V550, Jasco Inc.). Absorption changes were recorded at 440 nm (10 nm bandwidth, sampling rate 0.5 Hz). Absorption spectra and kinetic traces were analyzed by Spectra Analysis (Jasco Inc.) or Prism software (GraphPad Software Inc.). Rate constants were determined from linear fits of absorption changes recorded between 10 and 14 min after starting the reconstitution reaction. Kinetic constants were estimated using an extinction coefficient $\epsilon_{440\,nm}$ = 6,500 M^{-1} cm^{-1} for Isu1p, as deduced from the absorption spectrum of the purified, reconstituted protein (see Fig. 1*A*).

Rhodanese Aggregation Assays—Rhodanese from bovine liver (Sigma) was incubated at 38 μM in denaturation solution (6 M guanidine hydrochloride, 40 mM HEPES, pH 7.5, 20 mM KCl, 5 mM dithiothreitol) at 25 °C for 1 h. Denatured rhodanese was diluted to 2 μM in 400 μl of assay buffer (40 mM HEPES, pH 7.5, 20 mM KCl, 2,5 mM $MgCl_2$) and mixed rapidly for 10 s, and its aggregation was monitored continuously for 15 min by measuring the light scattering of the samples at 500 nm in a luminescence spectrometer (model LS-50B; PerkinElmer Life Sciences) at 25 °C.

Miscellaneous Methods—The following published methods were used: manipulation of DNA and PCR (41), transformation of yeast cells (42), isolation of yeast mitochondria and post-mitochondrial supernatant (43), labeling of yeast cells with radioactive iron (^{55}Fe), and the determination of iron incorporation by immunoprecipitation and liquid scintillation counting (5, 20). Cysteine desulfurase activity measurements were carried out under the conditions of the Fe/S cluster reconstitution assays and were subsequently quantified as described previously (44). For determination of sulfide formation of isolated mitochondria, 100 μg of organelles from cells grown in rich medium (yeast extract/peptone + 2% dextrose) were lysed in buffer A with 0.24% (w/v) dodecyl maltoside for 2 min on ice. Lysates were diluted 10-fold with buffer A, supplemented with 1 mM ATP, and incubated for 30 min at 30 °C. For each sample, the value obtained in the absence of cysteine was subtracted.

RESULTS

Spectroscopic Detection of Fe/S Cluster Formation on Isu1p in Vitro—For a kinetic analysis of Fe/S cluster assembly on Isu1p *in vitro*, we took advantage of the spectral changes associated with the formation of Fe/S holoproteins, which typically show absorption maxima around 420 nm (Fig. 1*A*; see Ref. 40). In our experimental setup, sulfide was supplied by anaerobically preincubating samples with the cysteine desulfurase Nfs1p for 20 min prior to the addition of apo-Isu1p. Fig. 1*A* shows the visible part of the absorption spectra of reconstitution mixtures containing 14 μM Isu1p at the start and after 3 h of incubation at room temperature under anaerobic conditions in the presence of 0.8 μM Nfs1p. The difference of these two spectra was virtually indistinguishable from the UV-visible spectrum of *S. cerevisiae* holo-Isu1p that was re-purified after an overnight reconstitution under the same conditions (Fig. 1*A*, compare difference spectrum and spectrum in *inset*) and to that of Isu1p obtained after reconstitution by chemical methods (data not shown). These observations demonstrate that the large majority of the optical changes that developed during this time interval were due to Fe/S cluster formation on Isu1p. This result was consistent with similar experiments carried out previously with bacterial IscU proteins *in vitro* (19, 40, 45, 46).

Absorption changes associated with Fe/S cluster formation on Isu1p were recorded over time at 440 nm, *i.e.* close to the maximum absorption of holo-Isu1p in the visible region. This wavelength was chosen, since it corresponds to the isosbestic point of short-lived initial absorption change of low amplitude that was consistently observed with reaction mixtures lacking Isu1p (see supplemental Fig. S1*A*). In the presence of Isu1p, a bi-phasic absorption increase was observed (Fig. 1*B*). The faster phase reached a plateau after ~5 min ($t_{1/2}$ ~ 90 s). While this reaction clearly required addition of Isu1p, it showed no consistent dependence on the Isu1p concentration and therefore was not studied

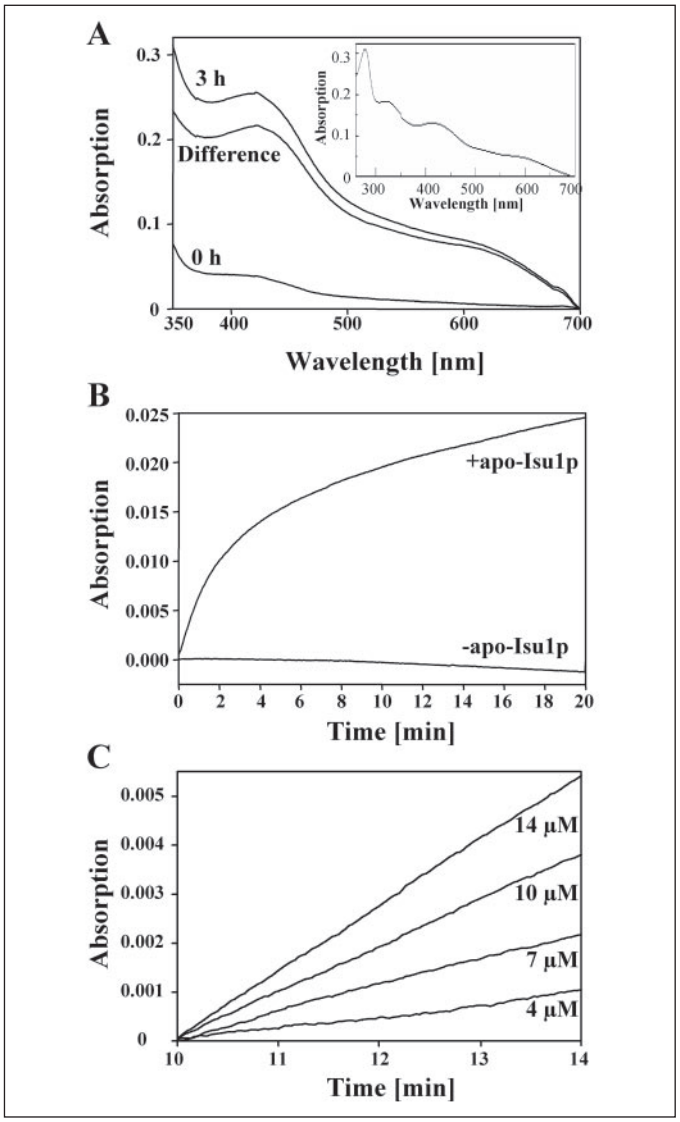

FIGURE 1. **Fe/S cluster formation on yeast Isu1p monitored by time-resolved spectroscopy *in vitro*.** *A*, absorption spectra were recorded using a standard reconstitution mixture containing 14 μM apo-Isu1p, 0.8 μM Nfs1p, 0.3 mM ferric ammonium citrate, and 4 mM cysteine immediately after addition of apo-Isu1p (0 h) and after 3 h. The difference spectrum was calculated. The *inset* shows the absorption spectrum of holo-Isu1p (17.1 μM) that was re-purified from a standard reconstitution mixture after an overnight incubation. Absorption maxima were observed at 320 and 416 nm. All spectra were adjusted to zero absorption at 700 nm. *B*, standard reconstitution mixtures containing 0.8 μM Nfs1p, 0.3 mM ferric ammonium citrate, and 4 mM cysteine were transferred to a double beam spectrometer under anaerobic conditions at room temperature. Absorption changes were recorded at 440 nm for 20 min in the absence or presence of 7 μM Isu1p. *C*, absorption changes were recorded at 440 nm between 10 and 14 min after addition of the indicated concentrations of Isu1p. All traces were normalized to zero absorption 10 min after starting the reaction.

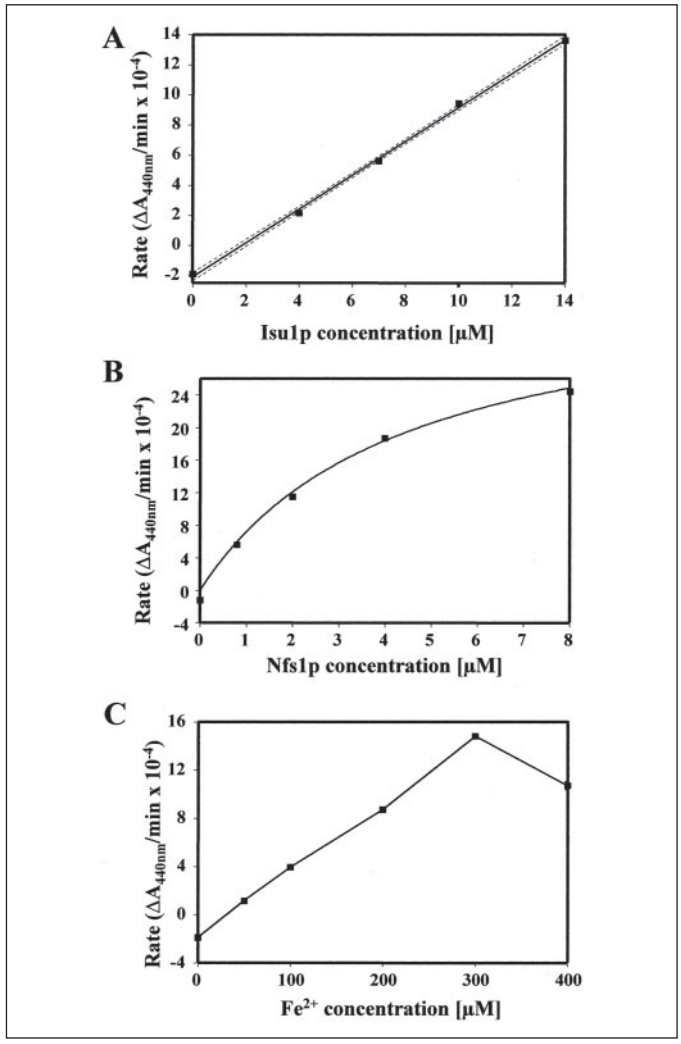

FIGURE 2. **Kinetic analysis of Fe/S cluster assembly on Isu1p *in vitro*.** *A*, rates of Fe/S cluster synthesis on Isu1p were calculated from linear fits of traces shown in Fig. 1*C* and plotted over the concentration of Isu1p. From the slope of the linear fit of the data an apparent second order rate constant of k_{app} = 21,600 ($\pm$300) M^{-1} min^{-1} was calculated. The *dashed lines* indicate the 95% confidence interval of the fit. *B*, Fe/S cluster synthesis was measured as in Fig. 1*C* in the presence of 7 μM Isu1p and various amounts of Nfs1p, and the calculated rates were plotted over the concentration of Nfs1p. The *solid line* shows a fit of the data according to the Michaelis-Menten equation. *C*, absorption changes were recorded in the presence of 7 μM Isu1p and increasing amounts of ferric ammonium citrate, and the calculated rates were plotted over the concentration of iron. The data shown were each taken from one representative set of experiment.

further (supplemental Fig. S1*B*). The second, slower phase was linear for at least 20 min (Fig. 1*B*), and its slope increased with increasing concentrations of Isu1p (Fig. 1*C*). The observed absorption increase was related to Fe/S cluster assembly on Isu1p (and not to an unbound Fe/S species), as it was neither observed in the absence of Isu1p nor in the presence of non-Fe/S proteins (Fig. 1*B* and supplemental Fig. S1*E*). In particular, the second (slower) phase reflected Fe/S cluster assembly on apo-Isu1p, as the rates of this reaction recorded at different wavelengths displayed a spectral behavior that was similar to the spectrum of reconstituted holo-Isu1p (supplemental Fig. S1*C* and S1*D*). In conclusion, the observed time-dependent absorption increase of the slower phase serves as a direct measure for studying Fe/S cluster formation on apo-Isu1p *in vitro* (19, 40, 45, 46).

To further characterize Fe/S cluster assembly on Isu1p, we studied the dependence of the rate of this reaction on added apo-Isu1p, sulfide, and Fe^{2+}. Reaction rates were determined from linear fits of the absorption changes occurring between 10 and 14 min after the addition of apo-Isu1p (see Fig. 1*C*), *i.e.* from a time interval in which the contribution of the initial fast phase to the total absorption change was negligible. The corresponding rates increased in a linear fashion with the concentration of added Isu1p (Fig. 2*A*). To obtain a practical measure for the velocity of the reconstitution process, we determined the apparent second order rate constant, k_{app} = 21,600 ($\pm$300) M^{-1} min^{-1}, (rate = k_{app} $\times$ [Isu1p] $\times$ [Nfs1p]) from the linear fit of the data in Fig. 1*C*. Thus, a sample containing 7 μM apo-Isu1p and 0.8 μM Nfs1p would produce holo-Isu1p at an initial rate of 0.12 μM per minute. As calculated from its optical density, the representative sample of Fig. 1*B* contained 3.7 μM

holo-Isu1p after 20 min. Of these 3.7 μM, 2.4 μM can be attributed to the slow, linear phase.

Next, we measured how increasing concentrations of the substrates sulfide (produced by Nfs1p) and Fe^{2+} influence the rates of Fe/S cluster production on apo-Isu1p. At high Nfs1p concentrations the velocity of the reaction approached a plateau (Fig. 2*B*). This behavior indicates a Michaelis-Menten kinetics in which sulfide produced by Nfs1p binds to an enzyme (presumably Isu1p) catalyzing cluster formation. Evaluation of the data of Fig. 2*B* according to the Michaelis-Menten formalism yielded the following parameters: K_m (Nfs1p) = 4.4 (±0.6) μM, v_{max} = 3.9 (±0.3) × 10^{-3}/min. Alternatively, the saturation behavior of Fe/S cluster synthesis may be explained by the inhibition of the reaction at high (toxic) sulfide concentrations. The discrimination between these two possibilities was not feasible because of the limiting amounts of Nfs1p. Finally, we determined the rates of Fe/S cluster formation on Isu1p at varying concentrations of Fe^{2+}. A virtually linear increase of the rates at low Fe^{2+} concentrations (<300 μM) was followed by a decline at higher concentrations (>300 μM; Fig. 2*C*). Most likely, these high amounts of Fe^{2+} are toxic and inhibit the Fe/S cluster reconstitution process. We therefore chose 300 μM Fe^{2+} as a standard concentration (40).

The Rate of Fe/S Cluster Reconstitution on Isu1p Is Increased by Chaperones—The central aim of our study was to investigate a possible function of the Hsp70 Ssq1p and the J-protein Jac1p in Fe/S cluster assembly on Isu1p. In our *in vitro* reconstitution assay the addition of a 2 μM concentration each of Ssq1p, Jac1p, and the nucleotide exchange factor Mge1p enhanced the rate of Fe/S cluster formation on Isu1p ~3-fold (Fig. 3*A*). This chaperone-induced rate increase was dependent on apo-Isu1p, as no absorption change was observed in the absence of this protein (supplemental Fig. S1*E*). Similar to the reaction in the absence of chaperones, a linear relationship between the rate of Fe/S cluster synthesis and the Isu1p concentration was again observed (Fig. 3*B*). The slope of the linear fit of the data was 2.5-fold higher than that of Fe/S cluster formation in the absence of Ssq1p, Jac1p, and Mge1p (*cf.* Fig. 2*A*). From the linear fit of the data an apparent second order rate constant of k_{app} = 54,200 ± 1,200 M^{-1} min^{-1} was calculated.

At first glance, these data suggested that the chaperones may improve the net efficiency of Fe/S cluster formation on Isu1p *in vitro*. However, we noted that although the stimulatory effect required Ssq1p, it was not dependent on the presence of Jac1p, despite its essential function in Fe/S protein maturation *in vivo* (Fig. 3*C*; Refs. 21–24). Moreover, increased amounts of Jac1p (10 μM instead of the standard 2 μM) did not further stimulate the rate of Fe/S cluster formation. This lack of a clear requirement of the essential co-chaperone Jac1p on Fe/S cluster formation on Isu1p strongly suggests that the majority of the stimulatory effects of Ssq1p were the likely result of an unspecific chaperone activity of Ssq1p *in vitro* rather than of its specific function in Fe/S protein biogenesis. Moreover, since interaction between Ssq1p, Jac1p, and Isu1p is essential for a proper catalytic cycle of Ssq1p *in vitro*, it seemed unlikely that the observed Ssq1p-dependent rate increase is mediated directly through Isu1p (24, 26, 33).

Because the effect of Ssq1 on Fe/S cluster formation seemed indirect, we proceeded to investigate the influence of the chaperones on the cysteine desulfurase activity of purified Nfs1p. Under the conditions of the Fe/S cluster reconstitution experiments, the presence of a 2 μM concentration each of Ssq1p, Jac1p, and Mge1p resulted in an ~5-fold increase in the amount of sulfide produced by Nfs1p (Fig. 4*A*, *bars 1* and *2*). The Nfs1p activity correlated in an almost linear fashion with the concentrations of Ssq1p, Jac1p, and Mge1p (Fig. 4*B*). The stimulatory effect of the chaperones on the Nfs1p activity was thus in the same range

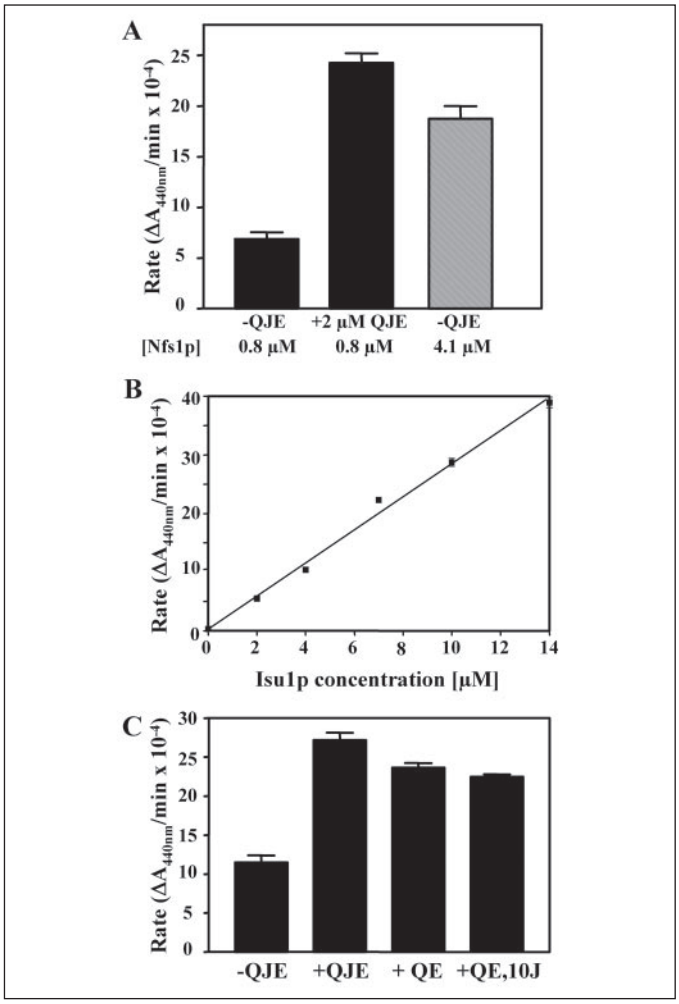

FIGURE 3. **The Ssq1p-induced rate increase of Fe/S cluster formation on Isu1p is independent of Jac1p.** *A*, rates of Fe/S cluster formation on Isu1p (7 μM) were determined from the absorption changes recorded at 440 nm between 10 and 14 min after initiation of the reaction in the absence (−*QJE*) or presence of 2 μM each of Ssq1p, Jac1p, and Mge1p (+*QJE*). The *striped bar* shows the rate of Fe/S cluster formation calculated from Fig. 2*B* for a sample lacking the chaperones but with the same cysteine desulfurase activity as a sample in which Nfs1p activity was measured in the presence of a 2 μM concentration each of Ssq1p, Jac1p, and Mge1p. *B*, absorption changes were recorded at 440 nm on standard reconstitution mixtures containing 0.8 μM Nfs1p, a 2 μM concentration each of Ssq1p, Mge1p, and Jac1p, and various amounts of Isu1p. From the slope of the linear fit of the data an apparent second order rate constant of k_2 = 54,200 (±1,200) M^{-1} min^{-1} was calculated. The data were taken from one representative set of experiment. *C*, rates of Fe/S cluster synthesis on apo-Isu1p (10 μM) were determined from the absorption changes at 440 nm in reaction mixtures containing 0.8 μM Nfs1p in the absence of chaperones (−*QJE*) and in the presence of a 2 μM concentration each of Ssq1p, Jac1p, and Mge1p (+*QJE*), a 2 μM concentration each of Ssq1p and Mge1p (+*QE*), or a 2 μM concentration each of Ssq1p and Mge1p and 10 μM Jac1p (*QE,10J*).

as their influence on the rate of Fe/S cluster formation on Isu1p. For a more quantitative assessment of the contribution of chaperone-induced Nfs1p activity to Fe/S cluster formation on Isu1p, we used the results of Fig. 2*B*. A chaperone-induced 5-fold higher Nfs1p activity (corresponding to 4.1 μM Nfs1p instead of the standard 0.8 μM) would produce a ~3.3-fold higher rate of Fe/S cluster formation (18 *versus* 5.5 × 10^{-4} min^{-1}). When this calculated rate was compared with the results presented in Fig. 3, *A* and *B*, the majority of the rate increase in Fe/S cluster formation on Isu1p could be explained by the effect on Nfs1p activity (see, *e.g.* Fig. 3*A*, *bar 3*). This finding suggests that the Ssq1p-induced rate increase on Fe/S cluster formation may be caused by an improved desulfurase activity of Nfs1p alone.

We next asked whether the stimulation of the Nfs1p enzyme activity by Ssq1p is of physiological relevance, by determining the requirements

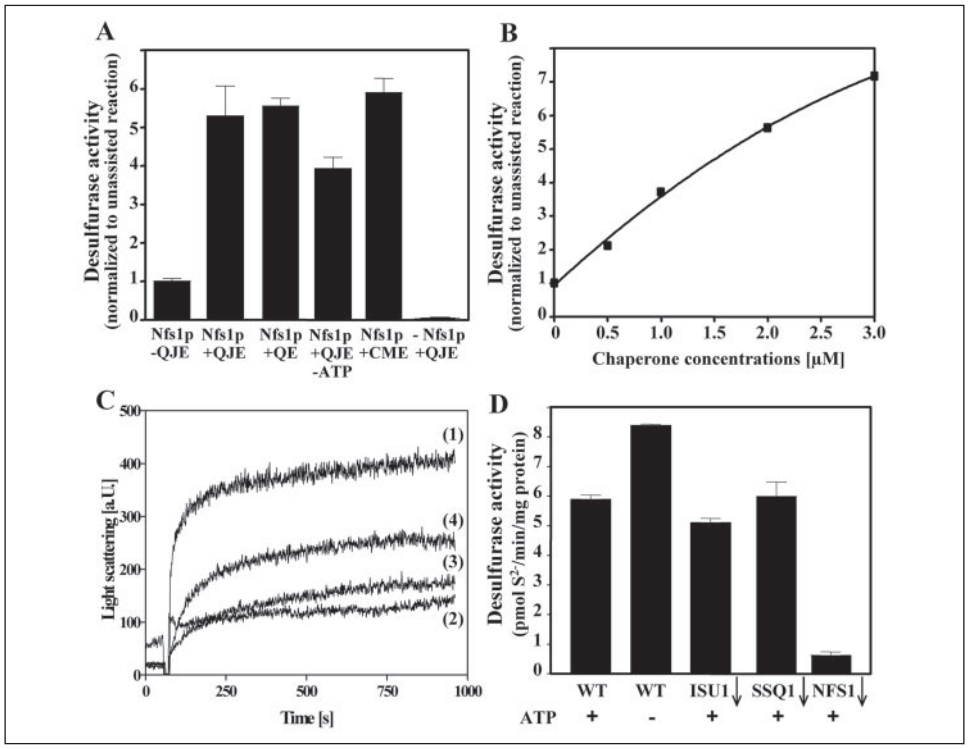

FIGURE 4. **The cysteine desulfurase activity of Nfs1p is stimulated by chaperones Ssq1p and Ssc1p *in vitro*.** *A*, Nfs1p (0.8 μM) was incubated in the absence of chaperones (−*QJE*) or in the presence of a 2 μM concentration each of Ssq1p, Jac1p, Mge1p (+*QJE*), a 2 μM concentration each of Ssq1p and Mge1p (+QE), or a 0.5 μM concentration each of Ssc1p, Mdj1p, and Mge1p (+*CME*), under experimental conditions as described in the legend to Fig. 1 with or without 0.25 mM ATP as indicated. After 20 min the formation of inorganic sulfide from cysteine was determined. Data are presented relative to the value observed in the sample lacking Ssq1p, Jac1p, and Mge1p. A control reaction containing a 2 μM concentration each of Ssq1p, Jac1p, and Mge1p, but lacking Nfs1p is included. *B*, Nfs1p (0.8 μM) was incubated with increasing stoichiometric amounts of Ssq1p, Jac1p, and Mge1p. The cysteine desulfurase activity of Nfs1p was determined as described for *B*. Data were normalized to the activity of a sample lacking chaperones. *C*, denatured rhodanese was diluted in sample buffer in the absence of Ssq1p (*trace 1*), in the presence of 2 μM Ssq1p (*trace 2*), 2 μM Ssq1p and 2 mM ADP (*trace 3*), and 2 μM Ssq1p and 2 mM ATP (*trace 4*). The precipitation of rhodanese was followed by recording the light scattering of the samples at 500 nm. *D*, mitochondria were isolated from wild-type (*WT*) yeast cells and the conditional strains Gal-ISU1/Δisu2 (*ISU1* ↓), Gal-SSQ1 (*SSQ1* ↓), and Gal-NFS1 (*NFS1* ↓) after growth in rich medium supplemented with glucose to repress expression of *SSQ1*, *ISU1*, and *NFS1* in the respective Gal strains. Mitochondrial detergent lysates were incubated for 30 min at 30 °C under the buffer conditions described in Fig. 1, and the formation of inorganic sulfide was determined subsequently.

for this enhancement of Nfs1p activity. First, the requirement of the co-chaperone Jac1p in this reaction was studied. The stimulation of the activity of purified Nfs1p remained virtually unchanged when either Jac1p or ATP was omitted (Fig. 4*A*, *bars 3* and *4*). The lack of dependence on a J-protein or ATP suggested that the stimulation of Nfs1p activity was most likely due to nonspecific interactions of Ssq1 with Nfs1 that prevent the unfolding of Nfs1p during the course of the reaction. To examine whether Ssq1p could nonspecifically interact with an unfolded protein and prevent aggregation, we utilized the protein rhodanese that is commonly used to test the ability of chaperones for such purposes (47, 48). As expected for a member of the Hsp70 family, Ssq1p was capable of protecting denatured rhodanese from aggregation *in vitro* (Fig. 4*C*). Ssq1p was somewhat more efficient at preventing aggregation of rhodanese in the absence of nucleotide or presence of ADP, consistent with the prolonged interaction of Hsp70 with protein substrate in the absence of ATP (31). We also tested whether the major mitochondrial Hsp70, Ssc1p, might also stimulate Nfs1p activity. The stimulatory effect of Ssc1p was even stronger than that of Ssq1p, as similar desulfurase activities could be observed at 4-fold lower concentrations of Ssc1p (Fig. 4*A*, *bars 2* and *5*). Since Ssc1p is at least 100-fold more abundant in mitochondria than Ssq1p (23), the stimulation of Nfs1p activity by Ssq1p *in vitro* is therefore most likely not relevant *in vivo*, as it is not dependent on Jac1p and ATP, and is performed more efficiently by Ssc1p.

To test this idea *in vivo*, we determined the cysteine desulfurase activities in mitochondria isolated from wild-type yeast cells or the regulatable mutants Gal-NFS1, Gal-SSQ1, and Gal-ISU1/Δisu2. In the latter strains, the endogenous promoters of the respective genes are replaced by the galactose-induced *GAL1–10* promoter that is repressed in the presence of glucose (20). If Nfs1p required Ssq1p function for its enzymatic activity, the cysteine desulfurase activity in mitochondrial detergent extracts should decline upon depletion of Ssq1p. In contrast to this prediction, mitochondrial lysates prepared from Ssq1p-depleted Gal-SSQ1 and Isu1p-depleted Gal-ISU1/Δisu2 cells showed wild-type levels of sulfide production in the presence of cysteine (Fig. 4*D*). Hardly any desulfurase activity was measured in mitochondria isolated from Gal-NFS1 cells depleted for Nfs1p, indicating that Nfs1p was solely responsible for the detectable cysteine desulfurase activity in mitochondrial lysates. Moreover, the desulfurase activities were independent of added ATP, indicating that the native Nfs1p in mitochondrial lysates did not require an ATP-dependent function of a chaperone. Taken together, these data clearly show that Nfs1p of yeast mitochondria does not require Ssq1p for desulfurase activity *in organello*. Therefore, the stimulation of the activity of purified Nfs1p *in vitro* by Ssq1p is most likely of no physiological relevance *in vivo*.

The Ssq1p Recognition Domain of Isu1p Is Not Required for Fe/S Cluster Formation on Isu1p—The data presented above suggested that the Ssq1p-induced rate increase for Fe/S cluster formation on apo-Isu1p *in vitro* was not mediated through Isu1p. To further investigate this issue, we took advantage of Isu1p mutants that carry single amino acid substitutions in the highly conserved Ssq1p binding motif comprised by residues PVK (27). These Isu1p mutant proteins have previously been

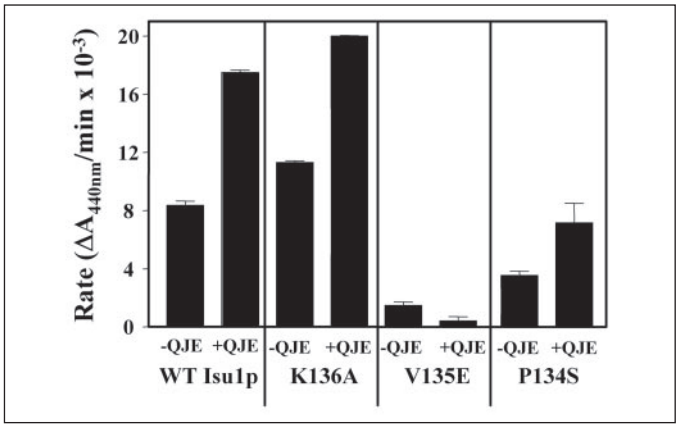

FIGURE 5. **Efficient *in vitro* Fe/S cluster synthesis on Isu1p carrying mutations in the Ssq1p recognition motif.** Wild-type (*WT*) Isu1p or the Isu1p point mutants K136A, V135E, and P134S (7 μM) were incubated under standard conditions in the presence (+*QJE*) or absence (−*QJE*) of a 2 μM concentration each of Ssq1p, Jac1p, and Mge1p. The rates of Fe/S cluster synthesis were determined from the absorption changes recorded at 440 nm between 10 and 14 min after initiation of the reaction. For experimental details see the legend to Fig. 1.

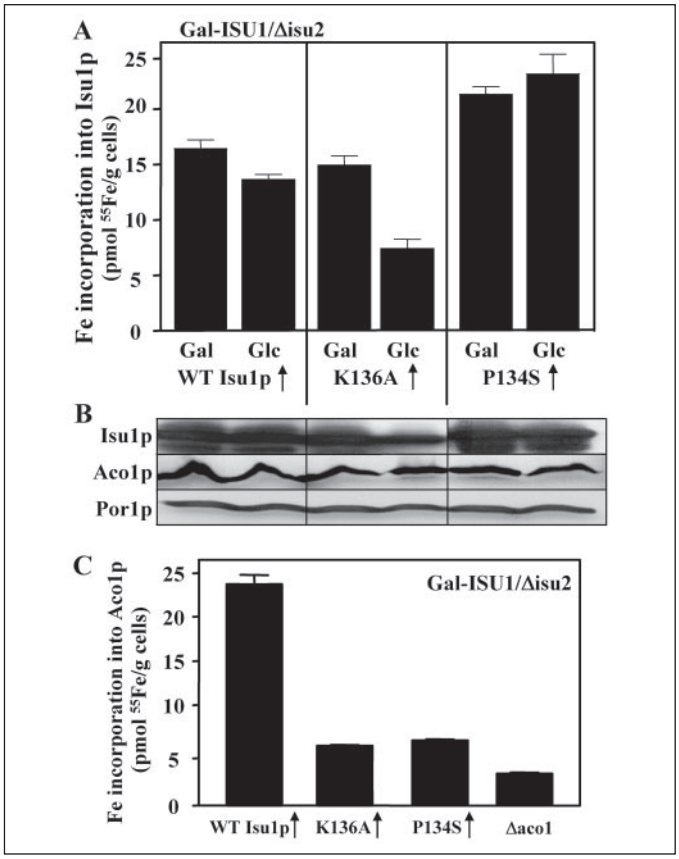

FIGURE 6. **Isu1p mutants with alterations in the Ssq1p recognition motif are capable of Fe/S cluster assembly *in vivo* but fail to rescue defects in cellular Fe/S protein maturation.** *A*, Gal-ISU1/Δisu2 cells overproducing (↑) wild-type Isu1p or the site-directed Isu1p mutants K136A and P134S were incubated in iron-poor minimal medium supplemented with galactose (*Gal*) or glucose (*Glc*) to produce or deplete endogenous Isu1p. Cells were radiolabeled with ^{55}Fe, Isu1p was immunoprecipitated from the cell lysates with Isu1p-specific antibodies, and the amount of co-immunoprecipitated ^{55}Fe was quantified by liquid scintillation counting. *B*, immunostaining of Isu1p, Aco1p, and porin in the extracts investigated in *A*. *C*, lysates were prepared from cells in *A* (grown with glucose), and the ^{55}Fe incorporation into mitochondrial Aco1p was determined by immunoprecipitation as described for *A*. Background ^{55}Fe incorporation was determined for cells lacking Aco1p (Δaco1 cells).

shown to exhibit a strongly reduced interaction with Ssq1p *in vitro*, to have a weak ability to stimulate the ATPase activity of Ssq1p, and to be unable to replace the wild-type Isu1p *in vivo* (27). Therefore, if the Ssq1p-assisted rate increase in Fe/S cluster formation on Isu1p would be due to a specific interaction between Isu1p and Ssq1p, this increase should not be observed with these mutants. We determined the rate of *in vitro* Fe/S cluster formation on three Isu1p point mutants, termed K136A, V135E, and P134S (27), in the absence and presence of 2 μM each of Ssq1p, Jac1p, and Mge1p. The mutant V135E apparently has lost its ability to bind an Fe/S cluster, in addition to the dramatically reduced binding affinities for Ssq1p, as little Fe/S cluster was formed (Fig. 5). In contrast, the mutants K136A and P134S were capable of supporting Fe/S cluster assembly in our *in vitro* assay. In the case of the K136A mutant, the observed rate was even slightly higher than that of wild-type Isu1p, while that of P134S was half of wild type. Addition of the chaperones increased the rates for both mutants by a factor of two, similarly as observed for wild-type Isu1p. Since an interaction between Ssq1p and these two Isu1p mutants is virtually undetectable (27), this observation strongly suggests that the chaperone-assisted rate increase cannot be attributed to a specific function of Ssq1p in this reaction.

We then explored whether the reduced interaction of the Isu1p mutants with Ssq1p had an impact on Fe/S cluster formation on Isu1p *in vivo*. To this end, we used an assay published earlier that follows the assembly of a radioactive Fe/S cluster on overproduced Isu1p by immunoprecipitation and scintillation counting (20). Wild-type and mutated *ISU1* alleles were inserted into the high copy yeast vector p426-GPD under the control of the strong constitutive *TDH3* promoter and transformed into the conditional yeast strain Gal-ISU1/Δisu2 that carries the endogenous *ISU1* gene under the control of the conditional *GAL1–10* promoter. In keeping with previous results (27), only the plasmid carrying the wild-type allele restored the growth defect of the Gal-ISU1/Δisu2 strain after depletion of endogenous Isu1p under repressive conditions (data not shown), even though the different plasmid-borne Isu1p forms were synthesized at high levels (Fig. 6*B*). For the determination of Fe/S cluster assembly on overproduced Isu1p, Gal-ISU1/Δisu2 cells containing the different *ISU1* plasmids were grown in iron-poor minimal medium in the presence of galactose and radiolabeled with ^{55}Fe. Subsequently, Isu1p was immunoprecipitated from cell lysates using specific antibodies. The amount of immunoprecipitated ^{55}Fe determined by scintillation counting served as a measure for Fe/S cluster assembly on Isu1p. Consistent with our *in vitro* analyses, ^{55}Fe was efficiently incorporated into both K136A and P134S, indicating that these mutations in the Ssq1p binding segment of Isu1p did not compromise Fe/S cluster assembly on Isu1p *in vivo* (Fig. 6*A*). Only background signal was obtained in the absence of overproduced Isu1p (data not shown; see Ref. 20).

We furthermore asked whether the different Isu1p mutant forms assembled their Fe/S cluster directly and thus still served as a *bona fide* scaffold protein or, alternatively, whether they received the Fe/S cluster from endogenous wild-type Isu1p. In the latter view, the mutant Isu proteins would behave as canonical target Fe/S apoproteins rather than as scaffolds. We therefore depleted the endogenous Isu1p by growth of the Gal-ISU1/Δisu2 cells in glucose-containing media and measured the ^{55}Fe incorporation into plasmid-borne Isu1p. Iron binding to the P134S mutant remained unchanged under these conditions (Fig. 6*A*). In case of the K136A mutant, iron binding was diminished 2-fold, a decrease that could be accounted for by the reduced levels of this protein under these conditions (Fig. 6*B*). To verify the successful depletion of endogenous Isu1p, we measured the incorporation of ^{55}Fe/S clusters into mitochondrial aconitase. Binding of ^{55}Fe to aconitase was strongly reduced in Isu1p-depleted Gal-ISU1/Δisu2 cells that carried the mutated *ISU1* alleles relative to cells expressing wild-type Isu1p (Fig.

6*C*). Apparently, the P134S and K136A mutant proteins were capable of binding an Fe/S cluster but unable to transfer this bound Fe/S cluster to target proteins. Taken together, these data provide additional *in vivo* evidence that the interaction between Isu1p and Ssq1p is not required for efficient Fe/S cluster formation on Isu1p. Yet, the failure of Ssq1p to interact with Isu1p impairs Fe/S cluster transfer to target Fe/S proteins.

DISCUSSION

The assembly of Fe/S proteins in mitochondria is a complex process involving more than ten proteins. Even though the assembly machinery is known for some time, the biochemical pathway of Fe/S cluster formation on apoproteins is still poorly defined. Most of the previous studies have been performed *in vivo*, and only few biochemical *in vitro* approaches have yet addressed the function of individual components. In this communication, we have analyzed the role the specific chaperone system, comprised of the Hsp70 Ssq1p, the co-chaperone Jac1p, and the nucleotide exchange factor Mge1p, in Fe/S cluster assembly on the Isu1p scaffold protein using a combination of *in vitro* and *in vivo* experiments. Previous studies on this chaperone system have concentrated on their protein interactions, the binding to their substrate Isu1p, and the catalytic cycle (21–24, 49). In the *in vitro* studies described here, we used a spectrometric assay that allowed the direct observation of Fe/S cluster formation on Isu1p by time-resolved spectroscopy. A similar approach has been employed recently to study the oxidative Fe/S cluster inversion of the bacterial oxygen sensor FNR from *E. coli* (50).

In the absence of the chaperones, the rate of the Fe/S cluster reconstitution showed a linear dependence on Isu1p concentration and a saturation behavior for added Nfs1p serving as the sulfur donor. This behavior is compatible with the idea that Isu1p functions as a scaffold on which the Fe/S cluster assembles. Whether this function involves an additional role of Isu1p in actively catalyzing Fe/S cluster assembly cannot be discriminated. The linear dependence of the reconstitution rates on the Isu1p concentration further indicates that Isu1p and Nfs1p do not undergo a rate-limiting complex formation during Fe/S cluster assembly, since such a complex would lead to a saturation behavior of the reaction rate. Our study therefore provides no explanation for the role of complexes between Isu1p and Nfs1p that were observed *in vitro* and *in vivo* (13, 51, 52). In addition, the observed reconstitution reaction is too slow to faithfully mimic the situation *in vivo*. This observation is similar to studies with bacterial Fe/S cluster assembly, which occurred at much slower rates than the *in vivo* process (18, 19, 53). We therefore conclude that an experimental system including only Nfs1p and Isu1p is too simple and additional components are required to better reflect the *in vivo* situation.

As a first set of additional components we tested the influence of the chaperone system. The formation of Fe/S cluster reconstitution on Isu1p was stimulated 3–5-fold by addition of Ssq1p. At first glimpse, this finding suggests a supportive role of the chaperone during the assembly of the Fe/S cluster on the scaffold protein. However, several lines of evidence indicate that the increased rates of Fe/S cluster assembly on Isu1p in the presence of Ssq1p resulted from a nonspecific function of Ssq1p on the Nfs1p enzyme activity *in vitro* rather than from a stimulation of cluster synthesis. First, Ssq1p enhanced the cysteine desulfurase activity of purified Nfs1p to virtually the same extent as the rate of Fe/S cluster formation on Isu1p. The increased sulfur production by an improved Nfs1p activity can therefore largely explain the higher reconstitution rates. Second, the rates of Fe/S cluster formation showed a linear dependence on the Isu1p concentration, similar to the reaction in the absence of chaperones. Apparently, complexes between the reaction components Isu1p and Ssq1p were either not formed or were not rate-limiting for Fe/S cluster synthesis on Isu1p. Third and most importantly, stimulation of the rate of Fe/S cluster formation on Isu1p by Ssq1p did not require Jac1p or the presence of ATP. Likewise, the stimulatory effect of Ssq1p on the desulfurase activity of Nfs1p was independent of Jac1p. An enhancement of the Hsp70 ATPase activity by its cognate J-chaperone is known to be essential for stabilization of the Hsp70-substrate interaction and for the overall turnover of the enzyme (24, 54). The lack of a specific contribution of Jac1p therefore indicates that the acceleration of Fe/S cluster formation on Isu1p was the result of a non-physiological chaperone activity of Ssq1p on isolated Nfs1p. This assumption is fully supported by our finding that Ssq1p is not needed for the formation and/or maintenance of active Nfs1p inside mitochondria. The cysteine desulfurase activity in isolated mitochondria remained unchanged upon depletion of Ssq1p and thus did not require this chaperone to gain or maintain its enzymatic function.

What might be the explanation for the *in vitro* effect of Ssq1p on Nfs1p activity? We have shown here that Ssq1p, similar to its bacterial counterpart HscA (47), can protect denatured rhodanese from aggregation. Therefore, Ssq1p might exhibit an effect on the folding state of isolated Nfs1p and thus stabilize it. This view was supported by the finding that Ssc1p, the major mitochondrial Hsp70-folding chaperone, also showed a stimulatory effect on Nfs1p function that was even more pronounced. In a living cell, the concentrations of Ssc1p are 100–1,000-fold higher than those of Ssq1p (23). Hence, even if this stabilization is physiologically relevant, it is rather unlikely that Ssq1p plays a major role in folding of mitochondrial proteins. Together, our *in vitro* analyses suggest that the Ssq1p/Jac1p/Mge1p chaperone system does not provide any detectable kinetic advantage for Fe/S cluster formation on Isu1p *in vitro* and thus may not play any role in this biosynthetic reaction.

This conclusion is further strengthened by using Isu1p mutant proteins with strongly reduced affinities for Ssq1p (27). The site-specific mutants carry single amino acid exchanges in the PVK segment, which is responsible for the interaction with Ssq1p. Strikingly, the stimulation of Fe/S cluster formation by Ssq1p on wild-type Isu1p was also observed with these mutants (K136A and P134S). This finding convincingly demonstrates that the accelerating effect of Ssq1p on this *in vitro* reaction cannot be attributed to a specific interaction between Isu1p and Ssq1p. Rather, as outlined above, the increased rate is caused indirectly through a non-physiological activation of Nfs1p by Ssq1p. These results were further corroborated by *in vivo* studies with these mutant Isu1p proteins building on a previous investigation that had demonstrated the assembly of an Fe/S cluster on Isu1p *in vivo* (20). Depletion of Ssq1p or Jac1p resulted in 3–5-fold higher amounts of Fe/S cluster on the Isu1p scaffold protein. This finding was taken to suggest a function of Ssq1p after cluster synthesis. We now report that the Isu1p mutant proteins K136A and P134S showed virtually unaltered efficiency of Fe/S cluster binding *in vivo*, despite their failure to undergo detectable interaction with Ssq1p. Nevertheless, these mutants were unable to support Fe/S cluster assembly on target apoproteins, as no rescue of the Fe/S cluster maturation defect of aconitase was observed by these proteins upon depletion of the endogenous Isu proteins in a Gal-ISU1/Δisu2 yeast strain. Apparently, the block of the Fe/S cluster assembly pathway occurs at a step following the synthesis of holo-Isu1p (20). In conclusion, an effective interaction between Isu1p and Ssq1p is not essential for holo-Isu1p formation arguing against a critical role of Ssq1p in this step.

While our study renders a function of Ssq1p in Fe/S cluster assembly on Isu1p unlikely, the exact molecular function within the process of Fe/S cluster biogenesis remains to be determined. The data reported here and in Muhlenhoff *et al.* (20) is most consistent with a role of Ssq1p

in transfer of an Fe/S cluster to a recipient apoprotein. However, it is also possible that the chaperones may function after a cycle of cluster transfer, altering the conformation of the scaffold to make it competent for assembly of another cluster on Isu1p. This second possibility is consistent with the fact that Ssq1p (and its bacterial homolog HscA) was shown to bind to both the apo- and holoforms of Isu1p (and IscU) (24, 33, 55, 56). At any rate, chaperones have no detectable influence on the *de novo* synthesis of an Fe/S cluster.

Isu1p specifically binds to both Ssq1p and Jac1p, and these interactions are highly conserved for the Hsp70s specialized for Fe/S cluster assembly (24–27, 56). However, not all Isu-type scaffolds may functionally interact with chaperones, as certain Isu-type proteins, such as those from *Thermotoga maritima*, lack the characteristic LPPVK binding motif for Ssq1/HscA-like Hsp70s and thus may represent a different class of IscU proteins that do not require chaperone action. In keeping with this idea, *T. maritima* lacks a close sequence correlate of Ssq1p. Recently, the influence of an Hsp70 chaperone on Fe/S cluster assembly on IscU and cluster transfer to an apoprotein has been investigated using DnaK and IscU from the bacterium *T. maritima* (17). This bacterial DnaK did not promote Fe/S cluster transfer from IscU to recipient apoferredoxin. Rather, the holoform of IscU was stabilized by DnaK thereby decreasing the rate of cluster transfer to apo-ferredoxin. It is unclear whether this effect is specific, as no dependence on DnaJ was shown or a non-physiological function of a molecular chaperone as described in this report.

Recent elegant studies showed that *E. coli* IscU or *Azotobacter vinelandii* NifU are capable of catalyzing Fe/S cluster transfer to target proteins without the need of further assistance of a chaperone (14, 18). Even multiple rounds of cluster transfer were documented. These and earlier *in vitro* studies suggest that Hsp70s are not necessarily required for Fe/S cluster transfer from IscU to apoproteins. However, the relatively low assembly rates *in vitro* suggest that the experimental conditions most likely do not faithfully mimic the *in vivo* situation. Thus chaperones, as well as additional ISC assembly proteins, may play a critical role in supporting efficient cluster transfer from the Isu/IscU scaffold. Our experimental *in vitro* system, in combination with *in vivo* studies using ISC protein-depleted mitochondria, may pave the way to gain further insights into the physiological mechanisms of these pathways.

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REFERENCES

1. Beinert, H., Holm, R. H., and Munck, E. (1997) *Science* **277,** 653–659
2. Beinert, H. (2000) *J. Biol. Inorg. Chem.* **5,** 2–15
3. Johnson, D. C., Dean, D. R., Smith, A. D., and Johnson, M. K. (2005) *Annu. Rev. Biochem.* **74,** 247–281
4. Lill, R., and Muhlenhoff, U. (2005) *Trends Biochem. Sci.* **30,** 133–141
5. Kispal, G., Csere, P., Prohl, C., and Lill, R. (1999) *EMBO J.* **18,** 3981–3989
6. Lill, R., and Kispal, G. (2000) *Trends Biochem. Sci.* **25,** 352–356
7. Frazzon, J., Fick, J. R., and Dean, D. R. (2002) *Biochem. Soc. Trans.* **30,** 680–685
8. Zheng, L., White, R. H., Cash, V. L., and Dean, D. R. (1994) *Biochemistry* **33,** 4714–4720
9. Zheng, L., and Dean, D. R. (1994) *J. Biol. Chem.* **269,** 18723–18726
10. Muhlenhoff, U., Balk, J., Richhardt, N., Kaiser, J. T., Sipos, K., Kispal, G., and Lill, R. (2004) *J. Biol. Chem.* **279,** 36906–36915
11. Schilke, B., Voisine, C., Beinert, H., and Craig, E. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 10206–10211
12. Garland, S. A., Hoff, K., Vickery, L. E., and Culotta, V. C. (1999) *J. Mol. Biol.* **294,** 897–907
13. Gerber, J., Muhlenhoff, U., and Lill, R. (2003) *EMBO Rep.* **4,** 906–911
14. Smith, A. D., Jameson, G. N., Dos Santos, P. C., Agar, J. N., Naik, S., Krebs, C., Frazzon, J., Dean, D. R., Huynh, B. H., and Johnson, M. K. (2005) *Biochemistry* **44,** 12955–12969
15. Ramelot, T. A., Cort, J. R., Goldsmith-Fischman, S., Kornhaber, G. J., Xiao, R., Shastry, R., Acton, T. B., Honig, B., Montelione, G. T., and Kennedy, M. A. (2004) *J. Mol. Biol.* **344,** 567–583
16. Dos Santos, P. C., Smith, A. D., Frazzon, J., Cash, V. L., Johnson, M. K., and Dean, D. R. (2004) *J. Biol. Chem.* **279,** 19705–19711
17. Wu, S. P., Mansy, S. S., and Cowan, J. A. (2005) *Biochemistry* **44,** 4284–4293
18. Bonomi, F., Iametti, S., Ta, D., and Vickery, L. E. (2005) *J. Biol. Chem.* **280,** 29513–29518
19. Wu, G., Mansy, S. S., Wu Sp, S. P., Surerus, K. K., Foster, M. W., and Cowan, J. A. (2002) *Biochemistry* **41,** 5024–5032
20. Muhlenhoff, U., Gerber, J., Richhardt, N., and Lill, R. (2003) *EMBO J.* **22,** 4815–4825
21. Voisine, C., Schilke, B., Ohlson, M., Beinert, H., Marszalek, J., and Craig, E. A. (2000) *Mol. Cell. Biol.* **20,** 3677–3684
22. Voisine, C., Cheng, Y. C., Ohlson, M., Schilke, B., Hoff, K., Beinert, H., Marszalek, J., and Craig, E. A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 1483–1488
23. Craig, E. A., and Marszalek, J. (2002) *Cell. Mol. Life Sci.* **59,** 1658–1665
24. Dutkiewicz, R., Schilke, B., Knieszner, H., Walter, W., Craig, E. A., and Marszalek, J. (2003) *J. Biol. Chem.* **278,** 29719–29727
25. Hoff, K. G., Ta, D. T., Tapley, T. L., Silberg, J. J., and Vickery, L. E. (2002) *J. Biol. Chem.* **277,** 27353–27359
26. Silberg, J. J., Hoff, K. G., Tapley, T. L., and Vickery, L. E. (2001) *J. Biol. Chem.* **276,** 1696–1700
27. Dutkiewicz, R., Schilke, B., Cheng, S., Knieszner, H., Craig, E. A., and Marszalek, J. (2004) *J. Biol. Chem.* **279,** 29167–29174
28. Knieszner, H., Schilke, B., Dutkiewicz, R., D'Silva, P., Cheng, S., Ohlson, M., Craig, E. A., and Marszalek, J. (2005) *J. Biol. Chem.* **280,** 28966–28972
29. Hoff, K. G., Cupp-Vickery, J. R., and Vickery, L. E. (2003) *J. Biol. Chem.* **278,** 37582–37589
30. Cupp-Vickery, J. R., Peterson, J. C., Ta, D. T., and Vickery, L. E. (2004) *J. Mol. Biol.* **342,** 1265–1278
31. Martin, J., and Hartl, F. U. (1997) *Curr. Opin. Struct. Biol.* **7,** 41–52
32. Hartl, F. U., and Hayer-Hartl, M. (2002) *Science* **295,** 1852–1858
33. Hoff, K. G., Silberg, J. J., and Vickery, L. E. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 7790–7795
34. Gerber, J., Neumann, K., Prohl, C., Muhlenhoff, U., and Lill, R. (2004) *Mol. Cell Biol.* **24,** 4848–4857
35. Sherman, F. (2002) *Methods Enzymol.* **350,** 3–41
36. Mumberg, D., Muller, R., and Funk, M. (1995) *Gene* (*Amst.*) **156,** 119–122
37. Bolliger, L., Deloche, O., Glick, B. S., Georgopoulos, C., Jeno, P., Kronidou, N., Horst, M., Morishima, N., and Schatz, G. (1994) *EMBO J.* **13,** 1998–2006
38. Kim, R., Saxena, S., Gordon, D. M., Pain, D., and Dancis, A. (2001) *J. Biol. Chem.* **276,** 17524–17532
39. Horst, M., Oppliger, W., Rospert, S., Schonfeld, H. J., Schatz, G., and Azem, A. (1997) *EMBO J.* **16,** 1842–1849
40. Agar, J. N., Krebs, C., Frazzon, J., Huynh, B. H., Dean, D. R., and Johnson, M. K. (2000) *Biochemistry* **39,** 7856–7862
41. Sambrook, J., and Russel, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
42. Gietz, R. D., and Woods, R. A. (2002) *Methods Enzymol.* **350,** 87–96
43. Diekert, K., de Kroon, A. I., Kispal, G., and Lill, R. (2001) *Methods Cell Biol.* **65,** 37–51
44. Papenbrock, J., and Schmidt, A. (2000) *Eur. J. Biochem.* **267,** 145–154
45. Smith, A. D., Agar, J. N., Johnson, K. A., Frazzon, J., Amster, I. J., Dean, D. R., and Johnson, M. K. (2001) *J. Am. Chem. Soc.* **123,** 11103–11104
46. Ding, H., and Clark, R. J. (2004) *Biochem. J.* **379,** 433–440
47. Silberg, J. J., Hoff, K. G., and Vickery, L. E. (1998) *J. Bacteriol.* **180,** 6617–6624
48. Mendoza, J. A., Rogers, E., Lorimer, G. H., and Horowitz, P. M. (1991) *J. Biol. Chem.* **266,** 13044–13049
49. Schmidt, S., Strub, A., Rottgers, K., Zufall, N., and Voos, W. (2001) *J. Mol. Biol.* **313,** 13–26
50. Sutton, V. R., Mettert, E. L., Beinert, H., and Kiley, P. J. (2004) *J. Bacteriol.* **186,** 8018–8025
51. Tokumoto, U., Nomura, S., Minami, Y., Mihara, H., Kato, S., Kurihara, T., Esaki, N., Kanazawa, H., Matsubara, H., and Takahashi, Y. (2002) *J. Biochem.* (*Tokyo*) **131,** 713–719
52. Agar, J. N., Zheng, L., Cash, V. L., Dean, D. R., and Johnson, M. K. (2000) *J. Am. Chem. Soc.* **122,** 2136–2137
53. Yuvaniyama, P., Agar, J. N., Cash, V. L., Johnson, M. K., and Dean, D. R. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 599–604
54. Silberg, J. J., and Vickery, L. E. (2000) *J. Biol. Chem.* **275,** 7779–7786
55. Johnson, D. C., Dos Santos, P. C., and Dean, D. R. (2005) *Biochem. Soc. Trans.* **33,** 90–93
56. Silberg, J. J., Tapley, T. L., Hoff, K. G., and Vickery, L. E. (2004) *J. Biol. Chem.* **279,** 53924–53931