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Fe–S Cluster Hsp70 Chaperones: The ATPase Cycle and Protein Interactions

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Abstract

Hsp70 chaperones and their obligatory J-protein cochaperones function together in many cellular processes. Via cycles of binding to short stretches of exposed amino acids on substrate proteins, Hsp70/J-protein chaperones not only facilitate protein folding but also drive intracellular protein transport, biogenesis of cellular structures, and disassembly of protein complexes. The biogenesis of iron–sulfur (Fe–S) clusters is one of the critical cellular processes that require Hsp70/J-protein action. Fe–S clusters are ubiquitous cofactors critical for activity of proteins performing diverse functions in, for example, metabolism, RNA/DNA transactions, and environmental sensing. This biogenesis process can be divided into two sequential steps: first, the assembly of an Fe–S cluster on a conserved scaffold protein, and second, the transfer of the cluster from the scaffold to a recipient protein. The second step involves Hsp70/J-protein chaperones. Via binding to the scaffold, chaperones enable cluster transfer to recipient proteins. In eukaryotic cells mitochondria have a key role in Fe–S cluster biogenesis. In this review, we focus on methods that enabled us to dissect protein interactions critical for the function of Hsp70/J-protein chaperones in the mitochondrial process of Fe–S cluster bio-genesis in the yeast *Saccharomyces cerevisiae*.

1. INTRODUCTION

As an Hsp70/J-protein system is key to the process of Fe–S cluster bio-genesis, the cycle of interaction of this class of chaperones with substrate proteins is described first. A brief overview of how the chaperone system fits into the process of Fe–S cluster biogenesis and a description of the complex evolutionary history of the chaperones involved in cluster biogenesis follows.

1.1 Substrate-Binding Cycle of Hsp70/J-Protein Chaperones

The ability of Hsp70s to perform their cellular functions depends on their cyclic interaction with substrates—short, hydrophobic stretches of residues in full-length proteins (Clerico, Tilitsky, Meng, & Gierasch, 2015; Kim, Hipp, Bracher, Hayer-Hartl, & Hartl, 2013). This cycle is allosterically regulated by nucleotide binding, hydrolysis, and exchange (Mayer, 2013). All members of the Hsp70 protein family have two domains, an N-terminal

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nucleotide-binding domain (NBD) of ~44kDa, with two lobes forming a deep pocket where adenine nucleotide binds; and a C-terminal substrate-binding domain (SBD) of ~26kDa, which consists of a β -sandwich subdomain (SBD β) forming a peptide-binding pocket and an α -helical subdomain (SBD α) forming a “lid” that covers the peptide-binding pocket (Fig. 1) (Kampinga & Craig, 2010). The two domains are joined by a flexible linker, which is important for interdomain allosteric communication.

In the ATP-bound state, the SBD and interdomain linker are docked onto the NBD. The α -helical lid (SBD α) and SBD β are restrained such that the peptide-binding pocket is not covered by the lid (Fig. 1). This “open” conformation allows for rapid binding and releasing of a substrate. Upon ATP hydrolysis to ADP, the conformation of Hsp70 changes dramatically. The SBD dissociates from the NBD and only the extended linker connects the two domains. Unrestrained, the α -helical lid covers the peptide-binding pocket of the SBD β , preventing dissociation of substrate. Most Hsp70s bind adenine nucleotides with high affinity. Therefore, a nucleotide exchange factor (NEF), which stimulates ADP release, is required to complete the substrate interaction cycle. Upon rebinding of ATP, which is normally in higher abundance than ADP in the cell, substrate is released, allowing the start of a new substrate interaction cycle (Mayer, 2013).

ATP hydrolysis is key to this cycle of Hsp70–substrate interaction. Because the intrinsic ATPase activity of Hsp70 is very low, a J-protein cochaperone, which stimulates ATPase activity, is obligatory. J-proteins are defined by the presence of a “J-domain” (Kampinga & Craig, 2010). This highly conserved domain consists of four α -helices, with two of them forming a finger-like structure connected by a loop region. This loop invariantly contains a His-Pro-Asp (HPD) tripeptide, critical for interaction with Hsp70. The J-domain interacts with ATP-bound Hsp70 and stimulates its ATPase activity. Although the molecular details of this transient interaction are not well understood, evidence suggest that the J-domain-binding interface is formed in the open conformation when the SBD and interdomain linker are docked onto the ATP-bound NBD (Mayer, 2013). Other domains of J-proteins are highly variable, in many cases displaying no sequence or structure similarities. Many J-proteins have a J-domain at their N-terminus followed by different types of domains that bind substrates, which allow them to interact with particular substrate proteins on their own, and as consequence to target substrate for Hsp70 binding (Craig & Marszalek, 2017). While J-proteins are obligatory, they only modestly stimulate Hsp70’s ATPase activity. Interaction with substrate results in the conformational change in the NBD that also, on its own, modestly increases ATPase activity. It is the synchronized interaction of the J-domain and the substrate protein that result in rapid hydrolysis, and thus the major conformational change that leads to the SBD dissociation from the NBD and closing of the α -helical lid over the substrate associated with the SBD β (Mayer, 2013).

1.2 An Overview of Mitochondrial Iron–Sulfur Cluster Biogenesis

Proteins involved in mitochondrial Fe–S cluster biogenesis were inherited from bacterial ancestors (Lill et al., 2015; Roche et al., 2013). The central component of both bacterial and mitochondrial pathways is a scaffold protein, termed Isu, on which an Fe–S cluster is assembled, before it is transferred to a recipient apo-protein. *Saccharomyces cerevisiae*

contains two paralogous, highly conserved and functionally redundant, scaffold proteins termed Isu1 and Isu2. The homologous scaffold in bacteria is termed IscU.

The process of Fe–S cluster biogenesis involves two sequential steps: cluster synthesis on the Isu scaffold and cluster transfer from Isu onto recipient apo-proteins (Fig. 2A). Each step engages a dedicated set of proteins forming multiprotein complexes (Beilschmidt & Puccio, 2014; Kim, Bothe, Alderson, & Markley, 2015; Rouault, 2015). Fe–S cluster assembly requires formation of the assembly complex composed of cysteine desulfurase interacting with the Isu scaffold and iron-binding protein, Yfh1, the yeast frataxin homolog (Prischi et al., 2010; Tsai & Barondeau, 2010). In mitochondria, cysteine desulfurase functions as a heterooligomer composed of the enzymatic subunit Nfs1, which provides sulfur needed for Fe–S cluster synthesis, and the tightly associated small accessory protein Isd11 (Richards & van der Giezen, 2006; Wiedemann et al., 2006). We refer to this complex as Nfs1 (Isd11) throughout. The biological role of Yfh1, which interacts with both Nfs1 and Isu, remains obscure (Pastore & Puccio, 2013; Stemmler, Lesuisse, Pain, & Dancis, 2010; Vaubel & Isaya, 2013). It has been implicated as an iron donor and/or as a regulatory subunit of the Fe–S cluster assembly complex (Bridwell-Rabb, Fox, Tsai, Winn, & Barondeau, 2014). Synthesis of the Fe–S cluster on the Isu scaffold also requires sulfur reduction by an electron transfer chain from NAD(P)H via ferredoxin reductase (Arh1) to ferredoxin (Yah1) (Webert et al., 2014; Yan, Adinolfi, & Pastore, 2015) (Fig. 2A). But, how these additional factors interact with the Fe–S assembly complex is not well understood.

The Fe–S cluster transfer from Isu scaffold onto recipient apo-proteins requires a dedicated Hsp70/J-protein chaperone system (Vickery & Cupp-Vickery, 2007). In mitochondria of *S. cerevisiae*, this system is composed of the Hsp70 Ssq1, its J-protein cochaperone called Hsc20 in most organisms (often called Jac1 in *S. cerevisiae*), and an NEF (Mge1 in *S. cerevisiae*) (Dutkiewicz et al., 2003). It is clear that the Isu scaffold is a substrate of Hsp70, and that the ATP-dependent formation of the Hsp70–Isu complex is directly responsible for Fe–S cluster transfer from Isu onto recipient protein (Fig. 2A) (Bonomi, Iametti, Morleo, Ta, & Vickery, 2008; Chandramouli & Johnson, 2006; Dutkiewicz et al., 2006). However, the exact molecular mechanism behind Hsp70 action during the Fe–S cluster transfer is not well understood.

1.3 Complex Evolutionary History of Mitochondrial Hsp70s Involved in Fe–S Cluster Biogenesis

Most proteins functioning in mitochondrial Fe–S cluster biogenesis were inherited from bacterial ancestors. Yet, the evolutionary history of the Hsp70s involved in this pathway is not simple (Schilke et al., 2006). In bacteria, including *Escherichia coli*, the Hsp70/J-protein chaperones functioning in Fe–S cluster biogenesis, termed HscA and Hsc20, respectively, are highly specialized and distantly related to other Hsp70/J-protein systems (Vickery & Cupp-Vickery, 2007). While the major bacterial Hsp70, DnaK, binds many different substrates and interacts with several J-protein cochaperones (Genevaux, Georgopoulos, & Kelley, 2007), HscA interacts exclusively with the Fe–S cluster scaffold and Hsc20 cochaperone. Moreover, in contrast to DnaK, HscA does not require an NEF to complete the substrate-binding cycle, as its low affinity for adenine nucleotide enables spontaneous ADP

to ATP exchange (Vickery & Cupp-Vickery, 2007). Mitochondria inherited the Fe–S cluster scaffold and the Hsc20 cochaperone from their bacterial ancestors, but specialized Hsp70 HscA was lost during evolution (Kominek, Marszalek, Neuveglise, Craig, & Williams, 2013; Schilke et al., 2006). As a result, many eukaryotic species, including humans, have only a single mitochondrial (mt)Hsp70, a descendant of DnaK, which binds the Isu scaffold and functions in the Fe–S cluster biogenesis with Hsc20 as cochaperone (Delewski et al., 2016; Pukszta et al., 2010).

Yet, this evolutionary story does not end here. A duplication of the mtHsp70 gene in a common ancestor of *S. cerevisiae* and closely related yeast species resulted in formation of an Hsp70 paralog, which became specialized in Fe–S cluster biogenesis (Kominek et al., 2013; Schilke et al., 2006). In the postduplication species one copy of the mtHsp70, termed Ssc1, remained multifunctional, while the other copy, termed Ssq1, functions exclusively in Fe–S cluster biogenesis. Therefore, although both *E. coli* and *S. cerevisiae* have specialized Hsp70s dedicated to Fe–S cluster biogenesis, their evolutionary origins are very different and therefore they can be considered as an example of convergent evolution at the biochemical level. Another interesting aspect, illustrated by this evolutionary story, is that both specialized and multifunctional Hsp70s can efficiently function in Fe–S cluster biogenesis, provided the specialized J-protein Hsc20 is available to target the Isu scaffold for Hsp70 binding. It is also important to stress that all key protein–protein interactions that are critical for the Hsp70/J-protein–Isu-binding cycle are conserved across phylogeny, regardless of whether a specialized or multifunctional Hsp70 is involved. Below we discuss these conserved interactions in more detail.

2. Hsc20–Isu INTERACTION

The Hsp70/Hsc20–Isu interaction cycle begins with formation of the Hsc20–Isu complex (Fig. 2A). Like many other J-proteins, Hsc20 interacts with substrate protein independently from its Hsp70 partner (Craig & Marszalek, 2017). Yet, the specificity of this interaction is quite unique, with Isu and Hsc20 forming a large binding interface. The interaction between Hsc20 and Isu is critical in vivo. Studies in yeast revealed that, when Hsc20 is severely defective in Isu-binding, cells are inviable; the presence of Hsc20 variants with diminished Isu affinity results in slow growth and reduced activity of mitochondrial enzymes (aconitase and succinate dehydrogenase) requiring Fe–S clusters for activity (Ciesielski et al., 2012).

On the C-terminal domain of Hsc20 (Fig. 2B), three highly conserved hydrophobic residues Leu¹⁰⁵, Leu¹⁰⁹, Tyr¹⁶³ and a patch of less conserved negatively charged residues (Asp¹¹⁰, Asp¹¹³, Glu¹¹⁴) are involved in the interaction with Isu (Andrew, Dutkiewicz, Knieszner, Craig, & Marszalek, 2006; Ciesielski et al., 2012; Fuzery, Oh, Ta, Vickery, & Markley, 2011; Fuzery et al., 2008). As revealed by amino acid replacement experiments, the hydrophobic residues play a key role, contributing greatly to the stability of the Hsc20–Isu complex. Despite having a lesser role in stability, the charged residues may have a role in providing specificity and orienting the interacting proteins. The complementary binding surface (Fig. 2B), on a three-stranded antiparallel β -sheet of the Isu scaffold, also consists of highly conserved hydrophobic residues (Leu⁶³, Val⁷², Phe⁹⁴), as well as a positively charged patch (Lys⁵⁴, Lys⁵⁵, Arg⁷⁴) (Kim et al., 2009; Majewska et al., 2013).

The interaction site of Hsc20 does not overlap with that of Hsp70. The Hsp70 binding site is the Leu¹³² Pro¹³³ Pro¹³⁴ Val¹³⁵ Lys¹³⁶ (LPPVK) pen-tapeptide on a distant flexible loop (Dutkiewicz et al., 2004; Hoff, Ta, Tapley, Silberg, & Vickery, 2002) (Fig. 2B). However, residues involved in the Isu–Hsc20 interaction are also critical for interaction of Isu with the cysteine desulfurase (i.e., *E. coli* IscS and *S. cerevisiae* Nfs1) present in the Fe–S cluster assembly complex (Majewska et al., 2013; Prischi et al., 2010). This raises a question of the possible physiological importance of overlapping binding sites for proteins having roles in the two different steps of Fe–S cluster biogenesis (i.e., Nfs1 in cluster assembly and Hsc20 in cluster transfer) (Fig. 2A). It is possible that sequential Isu interactions, first with Nfs1 and then with Hsc20, drive the ordered transition from assembly to transfer. One factor may be the relative affinities of Nfs1 and Hsc20 for the apo- and holo-form of Isu, with Nfs1 having higher affinity for the apo-form and Hsc20 for the holo-form. Hsc20 could also have a more direct role. The charged patch of Isu (K⁵⁴ K⁵⁵ R⁷⁴) (Fig. 2B) involved in the Hsc20–Isu interaction is not a part of the Nfs1-binding interface, suggesting that Hsc20 interaction might be initiated even when Isu is in the complex with Nfs1 (Majewska et al., 2013). This might allow Hsc20 to play a role of a kinetic switch between cluster assembly and transfer steps. Recent studies of bacterial Hsc20 indicating that it directly interacts with cysteine desulfurase (Puglisi, Yan, Adinolfi, & Pastore, 2016) provide further support for this attractive scenario. Moreover, Hsc20, as well as Nfs1, interaction protects Isu from proteolytic degradation by mitochondrial Lon protease (Pim1 in *S. cerevisiae*) both in vivo and in vitro (Ciesielski, Schilke, Marszalek, & Craig, 2016; Song, Marszalek, & Craig, 2012). These results underscore the importance of the Hsc20–Isu interaction, and indeed the complex network of Isu interactions, for both driving and coordinating Fe–S cluster biogenesis.

2.1 Glutathione-S-Transferase Pull-Down Assay

Many different techniques have been used to detect the Hsc20–Isu interaction, including: microcalorimetry, surface plasmon resonance (SPR) (Dutkiewicz et al., 2003; Hoff, Silberg, & Vickery, 2000), glycerol gradient centrifugation (Dutkiewicz et al., 2003), nuclear magnetic resonance spectroscopy (Kim et al., 2009), and glutathione-S-transferase (GST) pull-down assay (Ciesielski et al., 2012). In our hands the latter assay has worked very effectively. These assays are easy to set up and provide semi-quantitative results (Fig. 3), allowing us to test how replacements of residues involved in a given protein–protein interaction affect protein complex formation. We have also used this assay to analyze the competition between different proteins for binding to the same interaction partner (Fig. 3B). Below we present the procedure utilizing Isu1–GST fusion protein to detect proteins interacting with the Isu scaffold.

2.1.1 Equipment—No highly specialized equipment is necessary to perform GST pull-down assays. The specific equipment we typically use is listed below.

- Rotilabo-Block-Heater H250 for reaction mixture incubation
- Sigma 2–16K microcentrifuge
- Grant-bio PTR-30 rotator for bead incubation with rotation

- Standard SDS-PAGE equipment, 15% SDS-PAGE gels
- Epson Perfection 4990 PHOTO film scanner
- ImageJ quantification software

2.1.2 Buffers and Reagents

- Binding buffer (PB): 40mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid potassium salt) (HEPES-KOH, pH 7.5), 10% (v/v) glycerol, 100mM KCl, 1mM dithiothreitol, 10mM MgCl₂
- Laemmli sample buffer fourfold concentrated (SB): 0.125M Tris (hydroxymethyl)-aminomethane hydrochloride (Tris-HCl, pH 6.8), 20% (v/v) glycerol, 10% 2-mercaptoethanol, 5% SDS (sodium dodecyl sulfate), 0.004% bromophenol blue

2.1.3 Protein Preparations

- Isu1-GST (2.3mg/mL) protein purification was performed according to the procedure described in Ciesielski et al. (2012)
- Hsc20/Jac1 (4.9mg/mL) purified as described previously in Voisine et al. (2001), except that *E. coli* strain C41(DE3) was used for Hsc20/Jac1 expression
- Ssq1 (2.1mg/mL) protein purification was performed according to the procedure described in Manicki et al. (2014)

2.1.4 Procedure

- 1a. Equilibrate the reduced glutathione-immobilized agarose beads (Fluka, Sigma-Aldrich) by adding 210mg of dry beads to 40mL of buffer PB and incubate overnight at 4°C with rotation ~15rpm. Centrifuge swollen beads for 5min at $1000 \times g$.
- 1b. Wash beads three times with buffer PB (double volume of the beads), centrifuge between wash steps for 2min at $1000 \times g$.
- 1c. Incubate beads with PB buffer with 0.1% (v/v) Triton X-100 for 2.5h at 4°C with rotation ~15rpm. Centrifuge beads for 5min at $1000 \times g$.
- 1d. Wash beads three times with buffer PB (triple volume of the beads), centrifuge between wash steps for 2min at $1000 \times g$.
- 1e. Incubate beads with PB buffer with 0.1% (w/v) BSA (bovine serum albumin, Fluka, Sigma-Aldrich) added for 2.5h at 4°C with rotation ~15rpm. Wash beads three times with buffer PB (triple volume of the beads), centrifuge between wash steps for 2min $1000 \times g$. After extensive wash steps incubate resin in a refrigerator overnight.
- 1f. Finally, wash beads three times in buffer PB (double volume of the beads) and suspend in equal volume of buffer PB. Store refrigerated for, at most, 30 days.

2. In Eppendorf tube (500 μ L) set up a reaction mixture (150 μ L) consisting of buffer PB, Isu1–GST (2.5 μ M), Hsc20 (5 μ M). Incubate at 25°C for 15–30min in a thermo-block without rotation.
3. Take an aliquot of the reaction mixture (7.5 μ L) as a 5% “loading control,” add 7.5 μ L of twofold concentrated buffer SB, and store for further use.
4. To the remaining of the reaction mixture add 40 μ L of glutathione-beads (~20 μ L bead volume) and incubate at 4°C for 1h with rotation ~15rpm.
5. Wash beads one time with 500 μ L of buffer PB and then three times with 200 μ L of buffer PB. Centrifuge between wash steps at 1000 $\times g$ for 2min.
6. Suspend beads in 20 μ L of fourfold concentrated SB buffer, incubate for 10min at 90°C, load 15 μ L on SDS-PAGE 15% gel, and visualize by Coomassie Blue staining.
7. Image gel on film scanner and quantify bands by ImageJ or similar software.

2.1.5 Notes

1. As a control for nonspecific background binding, we substitute purified GST for Isu–GST in the reaction mixture. We process the control samples the same way as we do the experimental samples.
2. To control for both the amount of protein in the reaction mixture and the efficiency of the pull-down procedure we run “loading control” samples (see Section 2.1.4, step 3) on the same gel as we run the pull-down samples.

3. Hsp70/Hsc20–Isu-BINDING CYCLE

Isu scaffold interaction with the Hsp70/J-protein chaperones is critical for Fe–S cluster transfer from the scaffold to recipient apo-proteins, both in vivo and in vitro (Chandramouli & Johnson, 2006; Dutkiewicz et al., 2006; Vickery & Cupp-Vickery, 2007). For example, depletion of either Ssq1 or Hsc20 results in Fe–S cluster accumulation on the Isu scaffold and reduced activities of mitochondrial enzymes that require clusters for catalysis (aconitase, succinate dehydrogenase) (Dong et al., 2017; Muhlenhoff, Gerber, Richhardt, & Lill, 2003). These results demonstrate that the Hsp70/J-protein system is required for cluster transfer, but not for cluster synthesis on Isu. Moreover, biochemical experiments with purified bacterial HscA/Hsc20 chaperones demonstrated that efficient cluster transfer from the scaffold to recipient proteins such as apo-ferredoxin (Fdx) or apomonothiol glutaredoxin (Grx5) requires the presence of the chaperones (Bonomi et al., 2008; Bonomi, Iametti, Morleo, Ta, & Vickery, 2011; Chandramouli & Johnson, 2006; Shakamuri, Zhang, & Johnson, 2012). Consistent with the canonical Hsp70/J-protein substrate-binding cycle (Fig. 1) cluster transfer is strictly dependent on ATP hydrolysis. Below, we describe individual steps of the Hsp70/Hsc20–Isu-binding cycle and biochemical techniques that enabled us to study individual protein–protein interactions, as well as overall progression of the reaction cycle.

3.1 Hsp70–Isu Interaction

The binding site of Hsp70 on the Isu scaffold is the LPPVK pentapeptide localized on a flexible loop in close proximity (~8Å) away from the Fe–S cluster-binding site (Fig. 2B) (Dutkiewicz et al., 2004; Hoff et al., 2002). The LPPVK motif is evolutionary conserved; it is present in all orthologs of Isu, both prokaryotic and eukaryotic, which utilize an Hsp70/J-protein system for Fe–S cluster transfer onto recipient proteins. Biochemical evidence suggests that Hsp70s specialized in Fe–S cluster bio-genesis (i.e., *E. coli* HscA and *S. cerevisiae* Ssq1) recognize only this sequence. Yet, binding is via the same molecular mechanism as that of a typical, more promiscuous, Hsp70–peptide interactions (Cupp-Vickery, Peterson, Ta, & Vickery, 2004). The same conserved residues, in the peptide-binding pocket of the SBD β subdomain, are critical for HscA/Ssq1 interaction with Isu (Phe⁴⁶², Val⁴⁷²; see Fig. 2B) and DnaK interaction with substrate proteins (e.g., DnaK– σ^{32} interaction) (Hoff, Cupp-Vickery, & Vickery, 2003; Knieszner et al., 2005; Mayer et al., 2000; Tapley, Cupp-Vickery, & Vickery, 2005, 2006). In fact, the LPPVK sequence is very similar to the consensus peptide sequence recognized by DnaK (Rudiger, Germeroth, Schneider-Mergener, & Bukau, 1997).

Although the importance of the Hsp70–Isu interaction for the progression of the Fe–S cluster biogenesis is well established, the molecular mechanism directly responsible for the cluster transfer is not well understood (Bonomi et al., 2008; Chandramouli & Johnson, 2006; Shakamuri et al., 2012). One model suggests that the formation of the Hsp70–Isu complex affects the conformation of the Isu scaffold in such a way that it can no longer coordinate cluster binding, thus promoting cluster transfer onto recipient protein (Bonomi et al., 2011). Such a mechanism would be consistent with “remodeling” of protein structures by Hsp70 chaperones, such as Hsp70/J-protein-dependent disassembly of phage lambda DNA replication complex (Liberek, Georgopoulos, & Zylicz, 1988) or disassembly of clathrin-coated vesicles (De Los Rios & Goloubinoff, 2016; Sousa et al., 2016).

3.1.1 Detection of Ssq1–Isu Interaction Using GST Pull-Down Assay—

Interaction between Hsp70 and Isu has been detected previously (Cai et al., 2013; Dutkiewicz et al., 2003; Silberg, Hoff, Tapley, & Vickery, 2001). However, complex formation is very inefficient. This inefficiency is consistent with the canonical molecular mechanism of the Hsp70 substrate-binding cycle (Fig. 1) (Mayer, 2013). In the presence of ATP an open conformation of Hsp70 allows for rapid association, but also for rapid dissociation of Isu, while the closed lid of the ADP-bound conformation prevents efficient binding of Isu. However, very efficient formation of the Hsp70–Isu complex is observed in the presence of substoichiometric (catalytic) amounts of Hsc20 (Dutkiewicz et al., 2003; Hoff et al., 2000).

As depicted in Fig. 2, Hsc20 directly binds Isu and targets it for Hsp70 binding. The Hsc20–Isu complex interacts with ATP-bound Hsp70 allowing orchestration of the two critical interaction events: Isu interaction with the peptide-binding pocket in the SBD β subdomain, and J-domain of Hsc20 interaction with the ATP-bound Hsp70. The two interactions synergistically stimulate ATP hydrolysis to ADP, resulting in conformational changes of Hsp70 (Fig. 2B) that promote its stable interaction with Isu. Because Hsc20 dissociates from

the tripartite complex, upon ATP hydrolysis, it can interact with another Isu molecule. In consequence, over time, Hsp70–Isu complex accumulates in the reaction mixture. Hsc20-dependent formation of the Hsp70–Isu complex has been monitored by various biochemical assays: SPR and glycerol gradient centrifugation (Dutkiewicz et al., 2003; Hoff et al., 2000). Here we present results obtained using pull-down assay utilizing Isu1–GST fusion protein (Manicki et al., 2014). The procedure is essentially the same as described in Section 2.1 for Isu–GST interaction with Hsc20; however, the binding reaction is initiated by addition of ATP. This assay has also allowed us to test how individual amino acid substitutions in the intermolecular binding interfaces involved in the Hsp70/Hsc20–Isu interaction cycle affect the efficiency of Hsp70–Isu complex formation (Figs. 4 and 5).

3.2 ATPase Activity as a Measure of the Progression of the Hsp70/Hsc20–Isu Interaction Cycle

Similar to other members of the Hsp70 family chaperones specialized in Fe–S cluster biogenesis, HscA/Ssq1 possesses low, but measurable, ATPase activity, which is mildly stimulated in the presence of either Hsc20 or Isu (Dutkiewicz et al., 2003; Silberg, Tapley, Hoff, & Vickery, 2004; Silberg & Vickery, 2000). However, in the presence of both Hsc20 and Isu, this activity is stimulated over a hundred-fold. Because this stimulation depends on intermolecular interactions involved in the Hsp70/Hsc20–Isu-binding cycle, measurements of ATPase stimulation can be used to monitor the progression of the Isu-binding cycle. In this sense, the ATPase stimulation assay is complementary to the GST pull-down assays described above. In both cases, amino acid replacements that affect individual protein–protein interactions reduce assay output. It is worth noting, however, that for a given amino acid substitution variant a stronger negative effect is usually observed for the protein complex formation measured by GST pull-down than for ATPase stimulation (Ciesielski et al., 2012; Delewski et al., 2016; Majewska et al., 2013; Manicki et al., 2014). Therefore, although both assays can be applied for monitoring progression of the Hsp70/J-protein–Isu-binding cycle, ATPase stimulation is less sensitive to minor disruption of the protein–protein interactions.

Various assays have been used to measure the ATPase activity of Hsp70s involved in Fe–S cluster biogenesis (Dutkiewicz et al., 2003; Silberg et al., 2004; Silberg & Vickery, 2000). Here we present a method utilizing radioactive ATP (Fig. 6).

3.2.1 Equipment—No highly specialized equipment is needed to perform ATPase assay. The specific equipment we used is listed below.

- Rotilabo-Block-Heater H250 for sample incubation
- Sigma 1–15 microcentrifuge
- Beckman LS6000 scintillation counter
- Vortex

3.2.2 Buffers and Reagents

- ATPase reaction buffer (RB): 40mM HEPES–KOH, pH 7.5, 100mM KCl, 10% (v/v) glycerol, 1mM DTT, 10mM MgCl₂
- Stop buffer (ST): 1M perchloric acid, 1mM sodium phosphate

3.2.3 Protein Preparations

- Isu1 (1mg/mL) protein purification was performed according to the procedure described in Dutkiewicz et al. (2003)
- Mge1 (2.5mg/mL) purified as described in Azem et al. (1997)

3.2.4 Procedure

1. In an Eppendorf tube (1.5mL), set up a reaction mixture (12.5μL) consisting of buffer RB, Ssq1 (1μM), and other proteins as needed: Hsc20(0.5μM), Isu1 (10μM), and Mge1 (0.5μM). Start reaction by addition of radioactive ATP to a final concentration of 120μM [0.7μCi [γ -³³P]ATP (Hartmann Analytic GmbH, SCF-301, 2200Ci/mmol)]; [γ -³²P]ATP can also be used.
2. Incubate reaction mixture at 25°C in the thermo-block. Terminate reaction after 15min by the addition of 100μL of buffer ST. Keep the sample on ice.
3. Determine the total radioactivity in 5μL of sample after stopping reaction. Add aliquot to the scintillation cocktail (EcoLite™(+), ICN) and quantify radioactivity by liquid scintillation counting (Beckman LS6000).
4. Next, add 0.4mL of 20mM ammonium molybdate and 0.4mL of isopropyl acetate and mix the solution vigorously using vortex.
5. Separate the phases by centrifugation using microcentrifuge (1min at 15,000 × g).
6. Remove 150μL of the organic phase, containing the radioactive orthophosphate-molybdate complex, and determine the ³³P radioactivity by liquid scintillation counting, as described in step 3.

4. CONCLUDING REMARKS

Biogenesis of iron–sulfur clusters, similar to other cellular processes, is driven by multiprotein complexes that require specific protein–protein interactions for their assembly and function. Therefore, to gain mechanistic insight, one needs to dissect and validate those interactions. In this review, we have presented biochemical assays (Isu1–GST pull-down and ATPase stimulation of Hsp70) that allowed us to dissect interactions between the Isu scaffold and the Hsp70/J-protein chaperones involved in the Fe–S cluster biogenesis. However, besides the examples presented in this review, we have used GST pull-down assays to study other protein–protein interactions involved in the Fe–S cluster biogenesis. For example, by using Isu1–GST fusion protein, described here, we have also detected formation of the Fe–S assembly complex composed of Nfs1(Isd11)–Yfh1–Isu–GST. Moreover, we have used other GST fusion constructs, e.g., Yfh1–GST, Jac1–GST, to test

their interactions with protein partners. In all cases, results obtained by the GST pull-down assays enabled semi-quantitative interpretations of the effects. Moreover, recent technical developments allowed us to use GST fusion constructs to measure protein interactions in real time. We have recently accomplished this using the GST constructs described above immobilized to a biosensor of the Bio-layer Interferometer (BLI-Pall Corporation, USA) to measure interactions with their protein partners (data not shown).

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Fig. 1.

Overview of Hsp70–substrate interaction cycle. When ATP is bound to Hsp70 (left), substrate has easy access to the peptide-binding pocket in β -subdomain of SBD (SBD β). This is called the open or docked state, because the lid (SBD α), which traps the substrate by covering the binding pocket when ADP is bound (*right*), is restrained through interaction with the NBD, as are SBD β and interdomain linker. In concert with substrate in the binding pocket, J-domain, binding at the interface of NBD and SBD β , stimulates hydrolysis of ATP to ADP. Due to resulting conformational changes, the domains disengage, forming the undocked/closed state, stabilizing substrate interaction. Nucleotide exchange factor (NEF) completes the cycle (*bottom*). Brackets (*middle*) indicate dynamic transitions between ATP- and ADP-bound conformations. Note disengagement of the SBD α lid, but not SBD β , from NBD.

Fig. 2.

Fe-S cluster biogenesis in mitochondria of *Saccharomyces cerevisiae*. (A) Overview.

Assembly step: Fe-S cluster assembly requires interaction of the Isu scaffold with cysteine desulfurase Nfs1 (Isd11) and frataxin Yfh1. Together they form the assembly complex that catalyzes Fe-S cluster synthesis. Electrons needed for sulfur reduction are provided by the electron transfer chain via ferredoxin (Yah1) and ferredoxin reductase (Arh1). **Transfer step:** Following its assembly, the Fe-S cluster is transferred from the Isu scaffold to a recipient protein. Transfer requires the Hsp70 chaperone system consisting of Hsp70 Ssq1, J-protein cochaperone Hsc20, and nucleotide exchange factor Mge1. Hsc20 binds Isu forming the Hsc20-Isu complex; via its ability to compete with Nfs1 for Isu binding, Hsc20 has a key role in the transition between the assembly and the transfer steps. The Isu-Hsc20 complex interacts with ATP-bound Hsp70; orchestrated interaction of Isu and J-domain of Hsc20 with Hsp70 results in formation of a transient tripartite complex Ssq1-Isu-Hsc20 and stimulation of Ssq1's ATPase activity. Hydrolysis of ATP to ADP induces conformational changes of Hsp70 (Fig. 1), resulting in the formation of a stable Ssq1-Isu complex. As a result, the Fe-S cluster is transferred to a recipient protein. Isu is released from Ssq1, due to the nucleotide exchange activity of Mge1 protein, upon rebinding of ATP. A new cycle of Isu interaction then ensues. (B) Molecular models of Isu1, Hsc20, and Ssq1. Protein crystal structure of Hsc20 (Jac1 from *S. cerevisiae*, PDB code 3UO3, cyan). Residues involved in Isu1 interaction L¹⁰⁵, L¹⁰⁶, D¹¹⁰, D¹¹³, E¹¹⁴, Y¹⁶³ and H⁴⁸ P⁴⁹ D⁵⁰ motif of the J-domain critical for Hsp70 interaction are highlighted. Homology model of the Isu1 structure (orange, based on PDB code 3LVJ). Residues involved in Hsc20 interaction K⁵⁴, K⁵⁵, L⁶³, V⁷², R⁷⁴, F⁹⁴, and residues of the LPPVK motif (P¹³⁴, V¹³⁵, K¹³⁶) involved in Hsp70 binding are highlighted. Three cysteine residues (C⁶⁹, C⁹⁶, C¹³⁹) that coordinate the Fe-S cluster are marked in green. Homology models of the Ssq1 (green) in ADP-bound (top, based on PDB code 2KHO) and ATP-bound (bottom, based on PDB code 4JN4)

conformations. Residues of the peptide-binding pocket (F⁴⁶², V⁴⁷²) that interact with the LPPVK sequence of Isu, and residue R²⁰⁷ of the nucleotide-binding domain (NBD) involved in the interaction with J-domain HPD motif of Hsc20 are highlighted.

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Fig. 3.

Hsc20–Isu1 interaction. (A) Hsc20–Isu1 interaction detected by the GST pull-down assay. (Lanes 1–3) Isu1–GST (2.5 μ M) or GST (2.5 μ M) were incubated in the presence (+) or absence (–) of Hsc20 (5 μ M), as indicated. Glutathione resin was added to pull-down GST and associated proteins, which were separated by SDS-PAGE and stained with Coomassie Blue. (Lane 4) Five percent of the reaction mixture run on lane 1 was run as a loading control (load 5%). Notice strong Hsc20 signal (lane 1) when compared to the background signal (lane 3), which was obtained when Hsc20 was incubated with GST. “M” indicates the lane that has molecular weight markers. (B) Hsc20 competes with Nfs1(Isd11) for Isu1 binding. (Lanes 1–7) Isu1–GST (2.5 μ M) was mixed with Nfs1(Isd11) (5 μ M) and incubated for 15min to allow Nfs1(Isd11)–Isu1–GST complex formation. Incubation was continued for another 15min after addition of the Hsc20 competitor at the indicated concentrations. Glutathione resin was added to pull-down Isu1–GST complexes. (Lane 8) Five percent of the reaction mixture run in lane 7 was run as a loading control (load 5%). “*” indicates GST band that contaminates Isu1–GST protein preparation. Proteins were separated by SDS-PAGE, visualized by Coomassie Blue staining, and quantitated by densitometry. (C) Data from B were plotted as relative units (r.u.) with maximum binding of Nfs1 or Hsc20 set at 1. Notice decrease of the Nfs1 signal and increase of the Hsc20 signal with increasing concentrations of Hsc20 present in the reaction mixtures.

Fig. 4.

Hsp70-Isu interaction. (A) Detection of Ssq1-Isu1 interaction via GST pull-down assay. (Lanes 1–10) Isu1-GST (2.5 μ M) or GST (2.5 μ M) was incubated in the presence of ATP (2mM) with Ssq1 (5 μ M) and/or Hsc20 (0.25 μ M) as indicated. Glutathione resin was added to pull-down GST and associated proteins, which were separated by SDS-PAGE and stained with Coomassie Blue. (Lanes 11–12) Five percent of the reaction mixtures, which were run on lanes 3 and 2, were run as loading control (load 5%, L1 and L2, respectively). “M” indicates lane that has molecular weight markers. Notice strong Ssq1 signal (lane 2) when Isu1-GST wild type (WT) and Hsc20 were present in the reaction mixture. The Ssq1 signal was at the background level (lanes 4–5) when Hsc20 was absent (lane 1) or when Isu1-GST WT was replaced by the Isu1^{P134A/V135A/K136A}-GST (PVK) variant (lane 3), which is defective in Ssq1 interaction. (B) Hsc20 variants defective in Ssq1 interaction, Hsc20^{P134A} (P/A), or Isu1 binding, Hsc20^{L105A/L109A/Y163A} (LLY), inhibit formation of the Ssq1-Isu1 complex. (Lanes 1–4) Isu1-GST(2.5 μ M) or GST (2.5 μ M) was incubated in the presence of ATP (2mM) with Ssq1 (5 μ M) and Hsc20 (0.25 μ M) either wild type (WT), substitution variant (P/A), or (LLY), as indicated. Glutathione resin was added to pull-down GST and

associated proteins, which were separated by SDS-PAGE and stained with Coomassie Blue. (Lanes 5–7) Five percent of the reaction mixtures, which were run on lanes 1, 2, and 3, were run as loading control (load 5%, L1, L2, L3, respectively). “M” indicates lane that has molecular weight markers. Notice reduced Ssq1 signal when WT Hsc20 was replaced by substitution variants defective in interaction with Ssq1 (P/A) or with Isu1 (LLY).

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Fig. 5.

Substoichiometric amounts of Hsc20 are sufficient for Hsp70–Isu complex formation. (A) (Lanes 1–8) Isu1–GST (2.5 μ M) was mixed with Ssq1 (5 μ M) and varying concentrations of Hsc20, as indicated. Glutathione resin was added to pull-down GST and associated proteins, which were separated by SDS-PAGE and stained. (Lane 9) Five percent of the reaction mixture, which was run on lane 8, was run as loading control (load 5%). “*” indicates GST band that contaminates Isu1–GST protein preparation. (B) Densitometry values plotted as relative units (r.u.) with maximum binding of Ssq1 set at 1. Curve represents best fit to the data of Michaelis–Menten hyperbolic equation estimated by nonlinear regression using GraphPad Prism software. Notice that the Ssq1 signal increases with increasing concentrations of Hsc20 in the reaction mixture. Yet, the reaction is saturated at the substoichiometric concentrations of Hsc20 (0.125 μ M), consistent with the mechanism of the Hsp70/Hsc20–Isu interaction cycle (see Fig. 1).

Fig. 6.

ATPase activity of Hsp70 is stimulated in the presence of Isu and cochaperones. (A) Steady-state ATPase activity of Ssq1 was measured alone, or in the presence of indicated proteins. Bars represent average value for three repeated measurements with presented error bars as SD. (B) Stimulation of Ssq1 ATPase activity by Hsc20 in reaction containing Ssq1, Isu1, and Mge1, at indicated concentrations of Hsc20. Curve represents best fit to the data of Michaelis-Menten hyperbolic equation estimated by nonlinear regression using GraphPad Prism software. Notice synergistic stimulation of the ATPase activity of Ssq1 in the presence of cochaperones (Hsc20 and Mge1) and substrate (Isu1). Hsc20 stimulates the ATPase activity of Ssq1 at substoichiometric (0.125 μM) concentration consistent with the mechanism of the Hsp70/Hsc20 cyclic interaction with Isu (see Fig. 1). Also, notice that similar concentration of Hsc20 is needed to saturate both the ATPase of Ssq1 and formation of the Ssq1-Isu-GST complex (Fig. 5). Both reactions can be used to monitor the progression of the Hsp70/Hsc20-Isu interaction cycle.