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Molecular Chaperones HscA/Ssq1 and HscB/Jac1 and Their Roles in Iron-Sulfur Protein Maturation

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ABSTRACT Genetic and biochemical studies have led to the identification of several cellular pathways for the biosynthesis of iron-sulfur proteins in different organisms. The most broadly distributed and highly conserved system involves an Hsp70 chaperone and J-protein co-chaperone system that interacts with a scaffold-like protein involved in [FeS]-cluster preassembly. Specialized forms of Hsp70 and their co-chaperones have evolved in bacteria (HscA, HscB) and in certain fungi (Ssq1, Jac1), whereas most eukaryotes employ a multifunctional mitochondrial Hsp70 (mtHsp70) together with a specialized co-chaperone homologous to HscB/Jac1. HscA and Ssq1 have been shown to specifically bind to a conserved sequence present in the [FeS]-scaffold protein designated IscU in bacteria and Isu in fungi, and the crystal structure of a complex of a peptide containing the IscU recognition region bound to the HscA substrate binding domain has been determined. The interaction of IscU/Isu with HscA/Ssq1 is regulated by HscB/Jac1 which bind the scaffold protein to assist delivery to the chaperone and stabilize the chaperone-scaffold complex by enhancing chaperone ATPase activity. The crystal structure of HscB reveals that the N-terminal J-domain involved in regulation of HscA ATPase activity is similar to other J-proteins, whereas the C-terminal domain is unique and appears to mediate specific interactions with IscU. At the present time the exact function(s) of chaperone-[FeS]-scaffold interactions in iron-sulfur protein biosynthesis remain(s) to be established. *In vivo* and *in vitro* studies of yeast Ssq1 and Jac1 indicate that the chaperones are not required for [FeS]-cluster assembly on Isu. Recent *in vitro* studies using bacterial HscA, HscB and IscU have shown that the chaperones destabilize the IscU[FeS] complex and facilitate cluster delivery to an acceptor apo-protein consistent with a role in regulating cluster release and transfer. Additional genetic and biochemical studies are needed to extend these findings to mtHsp70 activities in higher eukaryotes.

KEYWORDS Hsp70, J-protein, IscU, Isu, structure, mitochondrial

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INTRODUCTION

Proteins containing iron-sulfur centers are ubiquitous and play essential roles in a wide range of redox, catalytic and regulatory processes in the cell.

The functions and properties of iron-sulfur proteins have been extensively characterized, but the mechanism and control of their formation and repair is only currently being elucidated. A combination of genetic and biochemical approaches have led to the identification of three types of systems involved in [FeS]-cluster assembly and maturation, and each contains a group of highly specialized proteins (see reviews by Johnson *et al.*, 2005; Mühlenhoff and Lill, 2006; Rouault and Tong, 2005). These include the *nif* system involved in maturation of nitrogenase in nitrogen fixing bacteria, the *suf* (sulfur utilization factor) system that functions under stress conditions in some bacteria and plays a general biosynthetic role in others, and the widely distributed *isc* (iron sulfur cluster) system that plays a general biosynthetic "housekeeping" role in both eubacteria and eukaryotes. In eubacteria the grouping of genes encoding many of the proteins into operons has facilitated their identification. The bacterial *isc* operon, for example, contains genes encoding a regulatory protein (IscR), a cysteine desulfurase (IscS), a scaffold protein for [FeS]-cluster preassembly (IscU), an alternate scaffold and/or iron-binding protein (IscA), a J-type co-chaperone (HscB) and Hsp70-class chaperone (HscA), and a [2Fe2 S]-ferredoxin (Fdx) (Figure 1). In eukaryotes, homologs of the *isc* components are found in mitochondria, and homologs of the *suf* components are found in plastids of photosynthetic organisms. The conservation of the bacterial *isc* machinery in eukaryotic mitochondria is consistent with the endosymbiotic origin of these organelles.

This review will focus on the Hsp70-type chaperones HscA from *Escherichia coli* and Ssq1 from *Saccharomyces cerevisiae* as well as their respective J-type co-chaperones HscB and Jac1. The role of Ssq1 and Jac1 in yeast mitochondrial iron-sulfur protein formation was reviewed by Craig and Marszalek (2002), and this article will cover the current state of our knowledge about both the eubacterial and yeast systems. It is assumed that insights gained from these specialized chaperone systems will also improve our understanding of the less extensively studied multifunctional mitochondrial Hsp70 (mtHsp70) and co-chaperone (HscB) that function in iron-sulfur protein biosynthesis of most higher eukaryotes.

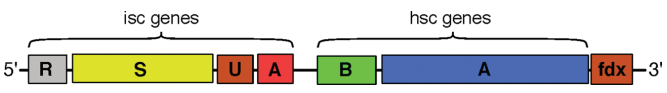

FIGURE 1 **Organization of eubacterial genes involved in iron-sulfur protein biogenesis.**

A SPECIALIZED CHAPERONE SYSTEM

Hsp70-family chaperone systems are widely distributed in eukaryotes, eubacteria, and many archaea and participate in diverse protein folding processes. Most organisms contain multiple forms of Hsp70s and auxiliary J-proteins, and these chaperones are found in nearly all cell compartments and organelles. In addition to their role in stress responses, Hsp70s are involved in normal housekeeping functions such as folding of newly synthesized proteins, cellular trafficking, and translocation of proteins across membranes, and control of regulatory proteins (reviewed in Mayer *et al.*, 2001; Mayer & Bukau, 2005). All of these activities depend on the ability of Hsp70s to bind reversibly to short, linear unfolded segments of polypeptide chains. Most Hsp70s are able to interact with a broad range of protein substrates, and recognition of these "client" proteins is determined by the intrinsic specificity of the Hsp70 and by J-type co-chaperone partners that assist in specific substrate delivery (reviewed in Fan *et al.*, 2003; Craig *et al.*, 2006). Binding of substrates to Hsp70s is also nucleotide-dependent, with the Hsp70·ATP complex (designated as the "Tense state") exhibiting lower affinity and faster exchange rates than the Hsp70·ADP complex (designated as the "Relaxed state") (Figure 2). Regulation of the rate of ATP hydrolysis thus affects both the extent to which substrates are bound and the lifetime of Hsp70-substrate complexes. J-protein co-chaperones, in addition to assisting substrate delivery, stimulate Hsp70 ATPase activity thereby stabilizing complex formation and facilitating selective substrate trapping.

Hsp70 chaperones have been highly conserved during evolution, and different isoforms exhibit a high degree of sequence conservation, suggesting that they have similar overall structural features (Karlin and Brocchieri, 1998). The N-terminal region, ≅45 kDa, comprises a nucleotide binding domain (NBD) which displays weak ATPase activity. A short linker region connects the NBD to a C-terminal segment, ≅25 kDa, which comprises a peptide or substrate binding domain (SBD). The intrinsic or basal ATPase activity of Hsp70s is usually low, <0.1 min^{-1}, but is generally stimulated several fold by substrate

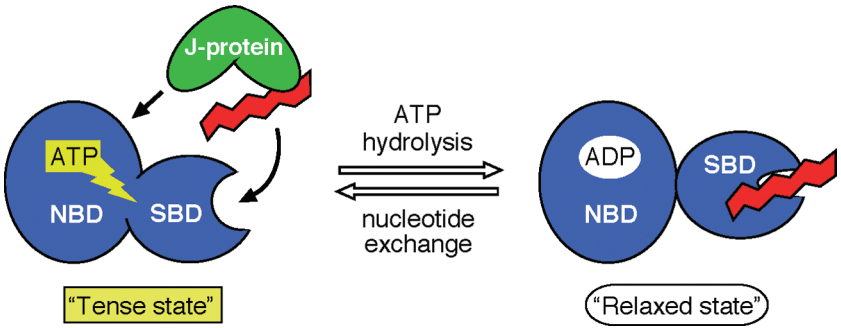

FIGURE 2 **Conformational states of Hsp70s and interactions with co-chaperones and substrates.**

or co-chaperone binding and may be synergistically enhanced up to ~1000-fold in the presence of both substrate and co-chaperone. This regulation prevents futile ATPase cycling of the chaperone in the absence of substrate and co-chaperone. The molecular mechanism of allosteric communication coupling ATPase activity in the NBD and polypeptide binding affinity of the SBD is not yet well understood. This is discussed in more detail below in the context of the HscA/Ssq1 and HscB/Jac1 systems.

Most Hsp70s display broad substrate specificity commensurate with a need to recognize a variety of proteins in general cell functions and/or stress responses. Indeed, it was initially believed prokaryotes contained a single Hsp70, designated DnaK, that served both stress-related and housekeeping functions (McKay, 1993). In 1994, however, a gene encoding a new Hsp70 homolog in *E. coli* was identified (Seaton and Vickery, 1994; Kawula and Lelivelt, 1994). This gene was located immediately upstream from and translationally coupled to the *fdx* gene encoding a [2Fe2S]-ferredoxin (Ta and Vickery, 1992; Ta *et al.*, 1992) and downstream from a gene encoding a novel J-protein (Kawula and Lelivelt, 1994). Expression of the Hsp70 gene product was found to be constitutive and not induced by heat shock, and the gene was designated as *hscA* (heat shock cognate). The predicted HscA protein (also designated as Hsc66; 66 kDa), exhibits only ≅40% sequence identity to DnaK and other Hsp70s. The upstream gene, designated *hscB*, encoded a novel J-protein. The predicted HscB protein (also designated as Hsc20; 20-kDa) contains an N-terminal J-domain and a novel C-terminal domain that is shorter and distinct from C-terminal domains found on Hsp40 J-type co-chaperones.

Initial studies on the general biochemical properties of *E. coli* HscA and HscB revealed that HscA exhibits a low basal ATPase activity (≅0.1 min^{-1} at 20°, ≅0.6 min^{-1} at 37°) typical of other Hsp70s (Vickery *et al.*, 1997; Silberg *et al.*, 1998). This intrinsic ATPase activity is stimulated four- to six-fold by HscB consistent with the role of HscB as a regulatory co-chaperone for HscA. DnaJ, the co-chaperone for DnaK, was able to stimulate HscA ATPase activity weakly, but supraphysiological concentrations were required suggesting that DnaJ does not normally function with HscA. In addition, HscB did not affect the ATPase activity of DnaK indicating a lack of "cross-talk" between the two chaperone systems. GrpE, a nucleotide exchange factor required for maximal activity of DnaK, did not affect HscA ATPase activity providing further evidence that the HscA/HscB and DnaK/DnaJ/GrpE chaperone systems have separate, non-overlapping cellular functions.

In vitro studies showed that HscA exhibited general chaperone activity as evidenced by the ability to suppress aggregation of model denatured substrate proteins (Silberg *et al.*, 1998). Furthermore, HscA chaperone activity was found to be nucleotide dependent consistent with ATP destabilization of HscA-substrate complexes typical of Hsp70-type chaperones. However, the endogenous physiological substrate(s) for HscA/HscB remained known. Studies by Hesterkamp and Bukau (1998) showed that lack of HscA does not lead to thermosensitivity or any major detectable defects in protein folding.

A ROLE IN IRON-SULFUR PROTEIN BIOGENESIS?

The function(s) of *E. coli* HscA and HscB were not immediately apparent and it was not initially known whether homologous proteins existed in other organisms. Shortly following their identification, however, determination of the complete genome sequences of

Haemophilus influenzae (Fleischmann *et al.*, 1995) and *E. coli* (Blattner *et al.*, 1997) revealed that the chaperones were encoded in a conserved region including several genes exhibiting similarity to those of the *nif* operon involved in maturation of [FeS]-clusters of nitrogenase (see above and Figure 1). This raised the intriguing possibility that HscA and HscB might have a specific role in the biogenesis of iron-sulfur proteins. Independent lines of work with bacteria and yeast provided key early support for this hypothesis. Dean and coworkers found that the diazotrophic bacterium *Azotobacter vinelandii* contained an *isc* operon similar to that of *H. influenzae* and *E. coli* (Figure 1) in addition to the *nif* operon (Zheng *et al.* 1998). Similarities of the *isc* components to *nif* components implicated in nitrogenase [FeS]-cluster formation suggested that the *isc* components might have housekeeping functions related to the general assembly and/or repair of other iron-sulfur proteins. Genetic constructs containing disruptions within the *isc* or *hsc* gene regions of *A. vinelandii* could not be stably maintained, consistent with a general role for the *isc* and *hsc* genes in cell metabolism and growth. Studies by Takahashi and coworkers with *E. coli* also provided support for a role of the *isc* and *hsc* components in bacterial iron-sulfur protein biogenesis. Increased yield of holo-forms of recombinant [2Fe2S] and [4Fe4S] proteins could be obtained when these were co-expressed with a plasmid containing the *isc* operon region (Nakamura *et al.*,1999), and inactivation of plasmid encoded *hscA* and to a lesser degree *hscB* was found to reduce the formation of recombinant iron-sulfur proteins (Takahashi and Nakamura, 1999). In a later study Tokumoto and Takahashi (2001) produced strains of *E. coli* in which individual chromosomal *isc* genes were disrupted. They found that inactivation of *hscA* or *hscB* reduced the activities of the iron-sulfur enzymes glutamate synthase and succinate dehydrogenase and overall growth rates. These studies suggested that the chaperone proteins HscA and HscB are necessary for efficient cluster assembly and/or delivery to acceptor proteins.

Coincident with the studies on bacterial *isc* and *hsc* components, new findings with the yeast *Saccharomyces cerevisiae* suggested that similar systems might be present in higher organisms. Sequencing of the genome of *S. cerevisiae* revealed the presence of genes encoding *isc*-like and *hsc*-like proteins (Goffeau *et al.*, 1996) suggesting that the pathway for biosynthesis of iron-sulfur proteins may have been conserved in eukaryotes. Experimental support for this idea came from studies by Culotta and colleagues during the characterization of mutants that suppressed oxidative damage in yeast deficient in superoxide dismutase (Strain *et al.*, 1998). One suppressor mutation occurred in a gene encoding an Hsp70 identified earlier as a mitochondrial protein whose absence caused cold sensitivity (Schilke *et al.*, 1996) and another occurred in a gene encoding a J-type co-chaperone homologous to bacterial HscB. The Hsp70 was designated Ssq1 (previously Ssh1), and the co-chaperone was designated Jac1 (J-type accessory chaperone). Based on the properties of its N-terminal signal sequence Jac1 was predicted to be localized to mitochondria together with Ssq1. Both *jac1* and *ssq1* mutants exhibited reduced levels of mitochondrial iron-sulfur enzymes aconitase and succinate dehydrogenase as well as impaired mitochondrial respiratory activity. This phenotype was similar to that of *nfs1* mutants defective in sulfur mobilization suggesting that Ssq1 and Jac1 might also participate in iron-sulfur protein biogenesis. The three *ssq1* mutants identified contained truncations in the region corresponding to the substrate binding domain of Hsp70s, and the *jac1* mutant contained a deletion of Asp-32, a conserved residue located in the J-domain (see below). The importance of Ssq1 in iron homeostasis was established independently by Dancis and colleagues who found that *ssq1* mutants (previously designated *ssc-2*) exhibit increased cellular iron uptake and mitochondrial iron accumulation (Knight *et al.*, 1998).

Subsequent work by several groups studying yeast provided additional evidence for a role of Ssq1 and Jac1 in iron-sulfur protein biogenesis. Craig and coworkers (Schilke *et al.*, 1999) also found that *SSQ1* mutants had low iron-sulfur enzyme activities and further observed a genetic interaction of *SSQ*1 with *ISU1*, a gene encoding a mitochondrial IscU homolog presumed to function as a [FeS]-scaffold assembly protein (Garland *et al.*, 1999). Haploid Δ*ssq1*/Δ*isu1* double mutants exhibited slower growth rates than Δ*ssq1* haploids, suggesting that the chaperone and [FeS]-scaffold protein likely function in a similar metabolic pathway. *JAC1* mutants were also confirmed to have reduced iron-sulfur enzyme activities consistent with a role in iron-sulfur protein formation (Voisine *et al.*, 2001; Lutz *et al.*, 2001; Kim *et al.*, 2001). The mature Jac1 protein was shown to be co-localized with Ssq1 in the mitochondrial matrix (Voisine *et al.*, 2001; Lutz *et al.*, 2001), and mutations in the J-domain region essential to Hsp70 interactions were found to

have deleterious effects consistent with its role as a co-chaperone (Voisine *et al.*, 2001). In addition, *jac1* mutants were observed to exhibit misregulation of iron uptake and increased mitochondrial iron accumulation (Kim *et al.*, 2001; Voisine *et al.*, 2001) as found for *ssq1* mutants (Knight *et al.*, 1998). Growth of *ssq1* and *jac1* mutants under low iron conditions to prevent excess iron accumulation further revealed that the observed reduction in iron-sulfur enzyme activities was not a consequence of high iron level. This suggested that the chaperones are involved in a cellular process essential to maintaining normal iron enzyme activities as might be expected for a role in iron-sulfur protein biogenesis (Voisine *et al.*, 2001).

Functional Convergent Evolution?

The *isc* and *hsc* components of bacteria and yeast were found to exhibit many similarities, but evolutionary and functional differences between the Hsp70 chaperone components soon became apparent. Analysis of phylogenetic distributions by Huynen *et al.* (2001) revealed that Ssq1 is more closely related to DnaK than to HscA suggesting that Ssq1 likely evolved from a generic DnaK-like Hsp70 rather than directly from HscA at some time during mitochondrial evolution. Thus a specialized Hsp70 having a role limited to iron-sulfur protein biogenesis may have arisen twice, once as HscA and its orthologs in bacteria and again as Ssq1 in certain fungi (Schilke *et al.*, 2006).

The different origins of HscA and Ssq1 are also reflected in their biochemical properties, especially with respect to nucleotide binding kinetics. In contrast to most Hsp70s *E. coli* HscA fails to bind to ATP-affinity columns suggesting an unusually low nucleotide binding affinity (Vickery *et al.*, 1997). Detailed studies on the kinetics of the HscA ATPase reaction cycle also revealed differences compared to other Hsp70s (Silberg and Vickery, 2000). The rates of dissociation of ATP and ADP were found to be much faster than for other Hsp70s and to result in lower nucleotide binding affinities. The rapid release of nucleotides from HscA is consistent with the apparent lack of the need for a nucleotide exchange factor (Silberg *et al.*, 1998) and with the structure of the NBD (discussed below). The rapid release of ADP and phosphate and the greater affinity for ATP compared to ADP·P_i suggest that the chaperone exists predominantly as the low affinity T-state HscA·ATP complex under cell growth conditions favoring normal energy charge (see Figure 2). Ssq1, in contrast, binds to ATP-affinity columns (Schmidt *et al.*, 2001) consistent with a high nucleotide binding affinity similar to other forms of Hsp70. Ssq1 was also found to interact with the mitochondrial nucleotide exchange factor Mge1 (Lutz *et al.*, 2001; Schmidt *et al.*, 2001) suggesting that the Ssq1 ATPase cycle is regulated in part by the rates of ADP release and ATP binding. Kinetic studies by Craig, Marszalek and coworkers confirmed that Ssq1 has a higher ATP affinity than HscA and requires Mge1 for maximal ATPase activity (Dutkiewicz *et al.*, 2003). These differences suggest that whereas the general function of HscA and Ssq1 in iron-sulfur protein biogenesis are likely to be similar the bacterial and yeast chaperones may be subject to different forms of regulation.

CHAPERONE [FeS]-SCAFFOLD INTERACTIONS

The Bacterial HscA/HscB/IscU System

The conserved linkage of *hscA* and *hscB* genes with *isc* genes in bacteria and the co-localization of yeast Ssq1 and Jac1 in mitochondria with *isc* homologues suggested that the chaperones might function in association with one or more of the *isc* gene products. The first experimental evidence for a specific cellular interaction came from studies carried out by Hoff *et al.* (2000) on the effects of bacterial *isc* components on HscA ATPase activity. Only the [FeS]-scaffold protein IscU was found to have an effect. IscU stimulated HscA ATPase activity approximately eight-fold above the basal level with half-maximal stimulation occurring at an IscU concentration $\cong$34 μM. In the presence of the co-chaperone HscB, however, a synergistic stimulation of about 400-fold was observed, and the K_m observed for IscU decreased to $\cong$2 μM. Both apo- and holo-[FeS]-forms of IscU gave similar stimulation of HscA ATPase suggesting that chaperone interactions could have effects on cluster assembly on the scaffold protein and/or transfer of clusters to acceptor proteins. Using surface plasmon resonance methods the binding of IscU to HscA could be observed directly, and HscB was found to enhance IscU binding to the HscA·ATP complex. IscU was also shown to interact with HscB using surface plasmon resonance and isothermal titration calorimetric techniques. Based on the binding affinities observed, the interaction of IscU with the

HscA and HscB appeared to be specific and to reflect a physiologically relevant interaction. In contrast to its effect on HscA, IscU elicited only a modest increase in DnaK ATPase activity (≅1.4-fold), and the stimulation was not further enhanced by HscB. Furthermore, DnaJ did not affect the HscA-IscU interaction, suggesting that the unique C-terminal region of HscB might be involved in IscU interactions and play a key role in the specificity of the system.

The findings that both HscA and HscB interact with IscU suggested that the scaffold protein might serve as a protein substrate for the chaperone system. Studies by Silberg *et al.* (2001) provided several lines of evidence in support of this hypothesis. These included a requirement for the substrate binding domain of HscA for ATPase activity stimulation, higher binding affinity of IscU to the R-state HscA·ADP complex, the presence of a single, high affinity (K_d ≅2 μM) binding site, and reduced binding affinity (K_d ≅26 μM) to a truncated form of HscA lacking the C-terminal α-helical "lid" region of the substrate binding domain (HscA:2–505; see below). These results were consistent with IscU binding as a native substrate to HscA and HscB suggesting that the chaperones may play a role in iron-sulfur protein biogenesis by regulating IscU[FeS] assembly or [Fe]-cluster transfer to acceptor apo-proteins.

The substrate specificity of HscA and the selective nature of the interaction between HscA and IscU were studied in more detail by Hoff *et al.* (2002). General peptide binding preferences of HscA were investigated by screening a heptameric peptide phage display library. HscA was found to favor peptides containing a core region of four nonpolar residues and especially those containing a central Pro-Pro motif. The location of the HscA binding site(s) on IscU was investigated using a cellulose-bound peptide array displaying the complete sequence of IscU as overlapping peptides 13 amino acids in length. HscA was found to selectively bind to a discrete region of IscU corresponding to residues 99 to 103 containing the amino acid sequence Leu-Pro-Pro-Val-Lys (Figure 3). The general chaperone DnaK, in contrast to HscA, was observed to bind to a number of IscU peptides underscoring the specificity of HscA and its selective interaction with IscU. A synthetic peptide corresponding to IscU residues 98 to 106 was able to stimulate the ATPase activity of HscA consistent with this peptide region playing a key role in HscA-IscU interactions. The LPPVK sequence

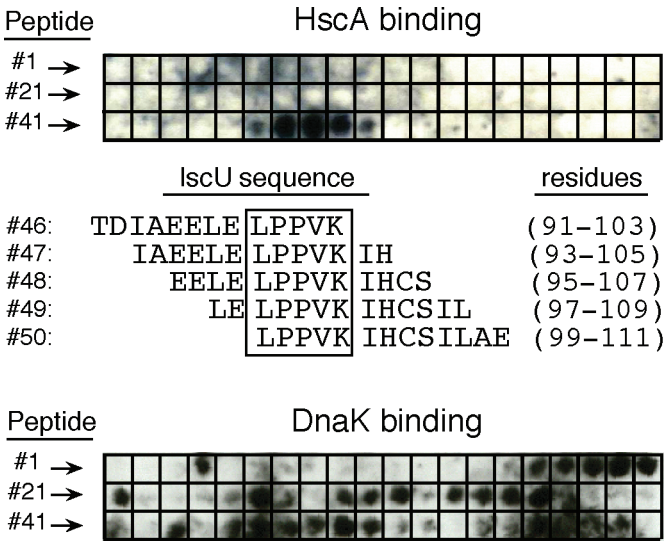

FIGURE 3 **HscA recognizes a specific region of IscU. Immunoblots of nitrocellulose arrays of overlapping peptides corresponding to the entire IscU primary sequence incubated with HscA (*upper panel*) or DnaK (*lower panel*) in buffer containing ADP. Sequences of peptides bound by HscA are aligned, and common residues are boxed (Hoff *et al.*, 2002).**

motif is conserved among IscU family members from both prokaryotes and eukaryotes suggesting that the mechanism of chaperone recognition and binding and the role of chaperone-scaffold interactions in [FeS]-cluster assembly and/or transfer has also been conserved during evolution.

The relative contributions of individual residues of the LPPVK motif of IscU to HscA binding and allosteric communication were investigated by Hoff *et al.* (2003). Studies using synthetic peptides suggested that the proline residue corresponding to Pro-101 was most critical for high affinity binding and for allosteric stimulation of HscA ATPase activity. Alanine scanning mutagenesis of the LPPVK region of IscU confirmed that Pro-101 was essential for both binding and ATPase activity enhancement and revealed that Val-102 and Lys-103 make lesser but significant contributions to complex stabilization. In ATPase stimulation assays HscB was found to enhance the apparent binding affinity of each of the mutants consistent with its role in binding IscU and targeting it to the HscA·ATP complex. Even in the presence of HscB, however, the IscU(P101A) and IscU(V102A) mutants exhibited a reduced level of synergistic ATPase enhancement suggesting that these residues may be critical for eliciting conformational changes required for allosteric communication between the substrate and nucleotide binding domains of HscA.

The Ssq1/Jac1/Isu1 System of Yeast

Studies of the yeast Ssq1/Jac1/Isu1 system yielded results similar to those of the bacterial HscA/HscB/IscU system. Dutkiewicz *et al.* (2003) found that while the ATPase activity of Ssq1 was not significantly affected by Isu1 alone cooperative stimulation (up to 12-fold) was observed in the presence of both Isu1 and Jac1. SPR studies also provided direct evidence for Isu1 binding to Jac1 and for Jac1 enhanced binding of Isu1 to Ssq1, findings similar to those with the HscA/HscB/IscU system (Hoff *et al.*, 2000). Interestingly, cooperative stimulation of Ssq1 ATPase activity was also found using bacterial IscU together with Jac1. This observation is consistent with the key role played by the conserved LPPVK motif in recognition by the chaperones and further suggests that the co-chaperone binding site (*i.e.*, for Jac1 and HscB) is likely to be conserved between yeast Isu1 and bacterial IscU (see also below). The importance of specific residues in the ^{132}LPPVK^{136} motif of Isu for interactions with Ssq1 also appear to be similar to those of IscU for interactions with HscA. Dutkiewicz *et al.* (2004) found that Isu1(P134A), Isu1(V135A), and Isu1(K136A) exhibited reduced affinity for Ssq1, but that binding to the Ssq1·ATP complex could be restored in the presence of Jac1. Isu1(P134A) and Isu1(K136A) also required higher concentrations to cooperatively stimulate Ssq1 ATPase activity consistent with reduced affinity for the Ssq1·ATP complex. Two additional mutants, Isu1(P134S) and the triple mutant Isu1(PVK->AAA), were more severely impaired in their ability to cooperatively stimulate Ssq1 ATPase activity. Thus, introduction of a polar residue at the central proline position or loss of nonpolar interactions at multiple sites coupled with loss of a possible electrostatic interaction severely affects the ability of Isu to bind and/or activate Ssq1 (see below). Importantly, Dutkiewicz *et al.* (2004) also found that the biochemical defects observed with the mutant Isu1 proteins correlated with growth phenotypes observed using cells expressing different mutant forms. These results provide strong evidence that the chaperone-scaffold interactions observed *in vitro* are also critical *in vivo*.

mtHsp70 s in Eukaryotes

Mitochondria of most eukaryotes contain a single multifunctional Hsp70 isoform (Leustek *et al.*, 1989; Mizzen *et al.*, 1989) that is homologous to mitochondrial Ssc1 of yeast (Craig *et al.*, 1989). This mtHsp70, also designated Grp75 and mortalin, is presumed to function in iron-sulfur protein formation together with an HscB/Jac1 co-chaperone. Craig and coworkers (Schilke *et al.*, 2006) have carried out studies in *S. cerevisiae* demonstrating that Ssc1 can substitute for Ssq1 and function with Jac1 in iron-sulfur protein biogenesis. Overexpression of Ssc1 or Jac1 was able to rescue growth phenotypes of Δ*ssq1* deletion mutants showing that the multifunctional isoform can function similarly to Ssq1. Ssc1 and Jac1 from *Yersinia lipolytica*, a fungal species lacking Ssq1, could also substitute for Ssq1 in *S. cerevisiae* suggesting that Ssc1 and Jac1 normally participate in iron-sulfur protein formation in that species. In addition, recombinant SBD fragments of mtHsp70 s from several species, including human mtHsp70, were shown to be able to bind to an Isu-derived peptide. These findings suggest that multifunctional mitochondrial Hsp70s with broad substrate specificity are likely to participate in iron-sulfur protein formation in organisms lacking a specialized isoform.

THE ATPASE REACTION CYCLE

Kinetic studies of the Hsp70 ATPase reaction cycle have provided key information about regulation of chaperone activity (reviewed in Mayer and Bukau, 2005), and investigations of the HscA and Ssq1 ATPase cycles have provided insight into their mechanism of action. Initial studies of HscA were carried out to determine the intrinsic rates of conversion of HscA between its different nucleotide-bound and conformational states (cf. Figure 2; Silberg *et al.*, 2000). HscA was found to interact with ATP in a two-step process that involved rapid nucleotide binding and subsequent conformational change involving conversion of HscA from the R- to the T-state. ATP hydrolysis occurs in the T-state, and this is followed by another conformational change that returns HscA to the R-state. ATP hydrolysis and the T->R conformational relaxation are rate-limiting in the overall cycle and are $>10^3$-fold slower than release of products ADP and phosphate. Thus, in contrast to other forms of hsp70 which are regulated at both ATP hydrolysis and ADP/ATP exchange, regulation of the HscA reaction cycle is expected to occur primarily at the hydrolysis step.

The interaction of HscB and IscU with different conformational states of HscA and the effects of the

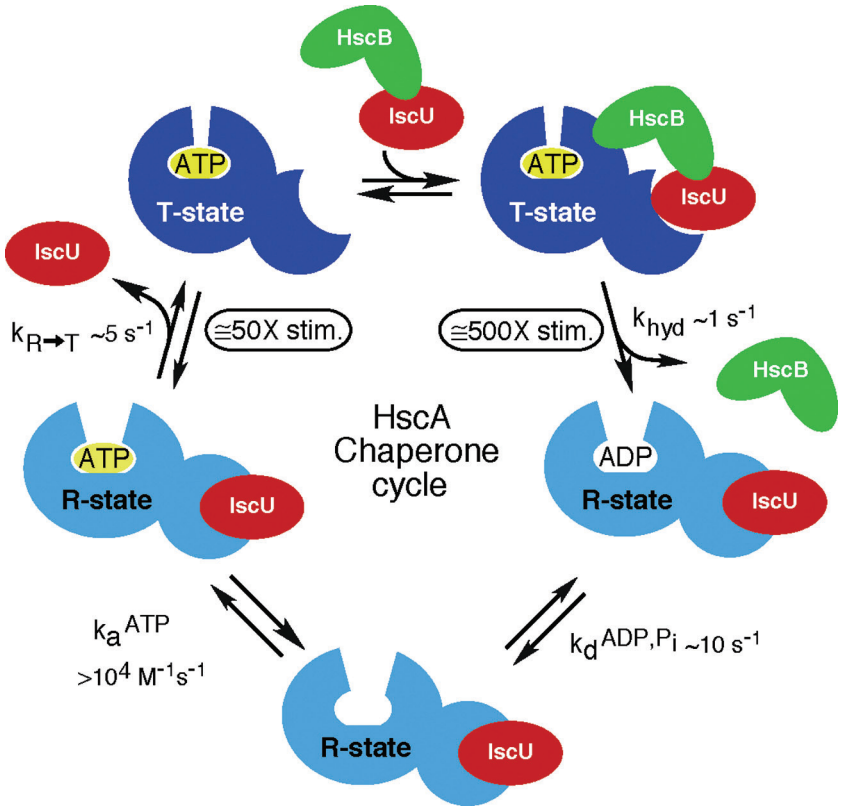

FIGURE 4 **The HscA chaperone cycle. Kinetic constants are from Silberg *et al.* (2004).**

co-chaperone and substrate on different kinetic steps of the ATPase cycle were investigated in more detail by Silberg *et al.* (2004). The findings are summarized in diagrammatic form in Figure 4. IscU binds to both ATP- and ADP-complexes of HscA, whereas HscB interacts only with the T-state ATP-complex. When present together IscU and HscB synergistically stimulate both ATP hydrolysis and conversion of HscA to the R-state leading to enhanced formation of the (HscA·ADP)-IscU complex, *i.e.*, substrate capture. ADP and phosphate are released relatively rapidly, such that there is no requirement for a nucleotide exchange factor. IscU was also found to increase the rate of conversion of the HscA·ATP complex to the low affinity T-state thereby favoring IscU release in the presence of ATP. The overall rate of the chaperone cycle is determined by the availability of the IscU-HscB substrate and co-chaperone complex.

The kinetics of the ATPase reaction cycle of Ssq1 have not been investigated in as much detail, but several differences are apparent. Craig, Marszalek, and coworkers (Dutkiewicz *et al.*, 2003) showed that Ssq1 has higher nucleotide binding affinity than HscA and requires the nucleotide exchange factor Mge1 for high steady state rates of ATP hydrolysis. In addition, the maximal synergistic stimulation of Ssq1 ATPase observed in the presence of saturating Isu1, Jac1, and Mge1 was only ≅12-fold. Thus, even with its slightly elevated intrinsic ATPase activity (~3-fold greater than that of HscA), the maximal steady state turnover for Ssq1 is an order of magnitude slower than observed for HscA in the presence of saturating amounts of substrate and co-chaperone. The significance of this difference in maximal rate determined *in vitro* with respect to the function of Ssq1 and HscA in iron-sulfur protein biogenesis in the cell is not known.

CHAPERONE STRUCTURE

A full understanding of the role of HscA/Ssq1 and HscB/Jac1 interactions with IscU/Isu will require detailed structural information for the chaperone(s), the co-chaperone(s) and the substrate(s). In addition, structures of different conformational states of the chaperones, of both the apo- and holo-forms of IscU/Isu, and of the various complexes of the proteins will be necessary to reveal the effects the chaperone has on the scaffold protein, the [FeS]-cluster and

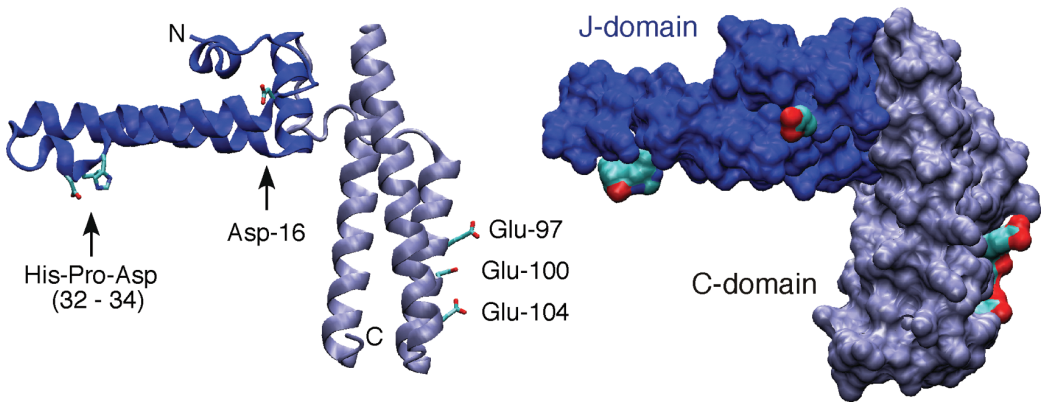

FIGURE 5 **Crystal structure of HscB. Ribbon and surface representation are shown with the J-domain (*blue*), C-domain (*gray*), and indicated residues by atom type (carbon, *cyan*; nitrogen, *blue*; oxygen, *red*). PDB coordinate file 1fpo (Cupp-Vickery and Vickery, 2000).**

how the process is regulated. Unfortunately, efforts to determine atomic resolution structures by x-ray crystallography have been hampered by difficulties in obtaining suitable diffraction quality crystals, and determination of solution structures by NMR methods is difficult due to the size and stability of the proteins and complexes. Additional biophysical approaches will likely be required to characterize some states and/or complexes and will also be useful for monitoring the kinetics of conformational changes at different stages of the reaction cycle. Progress to date on the individual components and some complexes is discussed below.

HscB

The crystal structure of the co-chaperone HscB from *E. coli* has been determined to a resolution of 1.8 Å (Figure 5; Cupp-Vickery and Vickery, 2000). The protein is folded into two distinct regions, an N-terminal J-domain (residues 1 to 75) connected by an eight residue loop to a C-terminal domain (84 to 171). The structure of the J-domain, involved in interactions with HscA, resembles J-domain fragments of the Hsp40 co-chaperones *E. coli* DnaJ and human Hdj1 previously determined by solution NMR methods. The C-terminal domain, implicated in binding and targeting proteins to HscA, is unique and consists of a three-helix bundle in which the two longer C-terminal helices comprise an anti-parallel coiled-coil. The J- and C-domains make contact through an extensive hydrophobic interface ($\cong$650 Å^2) suggesting that the relative positions and orientations of the two domains are fixed. The rigid structure suggests that HscB, in addition to enhancing the ATPase activity of HscA to trap IscU, may also function to facilitate positioning of the substrate protein on the chaperone.

The site of interaction of the J-domain of HscB with HscA is not known but is likely to involve residues of the conserved J-protein signature motif (His-Pro-Asp). Replacement of these residues in Jac1 with alanine disrupted its function consistent with a role in Ssq1 interactions (Voisine *et al.*, 2001). The homologous residues in HscB, ^{32}His-Pro-Asp^{34}, are located near the C-terminal end of helix-A and are exposed to solvent on the "lower" face of the J-domain. The structure also reveals the critical location of the ΔAsp-32 deletion in the *jac1–1* mutant identified in yeast (Strain *et al.*, 1998). This residue is conserved among J-proteins, and the side chain of Asp-16 in HscB is involved in H-bonding interactions with amide groups ("N-cap") of residues 18 and 19 at the beginning of the A-helix. Deletion of this aspartic acid residue would be expected to destabilize the protein consistent with the observed phenotypic properties (Strain *et al.*, 1998, Voisine *et al.*, 2001) and the reduced level of Jac1 protein found in *jac1–1* mutants (Voisine *et al.*, 2001).

The α-helical C-terminal domain of HscB is not present in other J-type co-chaperones and is presumed to mediate specificity for IscU binding (Hoff *et al.*, 2000). Analysis of conserved residues on the surface of the C-domain revealed a cluster of acidic residues and a nearby hydrophobic patch suggesting that this region might be involved in interactions with IscU. Andrew *et al.* (2006) tested this possibility by carrying out mutagenesis studies of the *JAC1* gene in yeast. Mutants in which homologous residues of Jac1 in this

region were replaced with alanine displayed a wild-type growth phenotype suggesting that interactions involving this region are not critical *in vivo*. *In vitro* studies, however, revealed that a mutant containing six alanine replacements did exhibit reduced Isu1 binding affinity and required higher concentrations for Jac1-mediated stimulation of Ssq1 ATPase activity. These results are consistent with Isu1 interacting with Jac1 in this region, but suggest that independent interactions with Ssq1 are sufficient to meet yeast growth requirements. The importance of scaffold-co-chaperone interactions in other systems has not been investigated.

HscA Modeling and Solution Studies

Structures of HscA and of its IscU complex have not been determined, and initial efforts to understand the molecular basis of HscA-IscU interactions employed homology modeling. Hoff *et al.* (2003) generated a model of the SBD of HscA based on the crystal structure of a DnaK(SBD)-peptide complex (Zhu *et al.*, 1996). A peptide corresponding to IscU residues ^{98}ELPPVKI^{104} was modeled into the substrate binding cleft in an extended conformation and orientation similar to that observed for the NRLLLTG peptide in the DnaK(SBD) complex. This model was subjected to energy minimization and simulated annealing, and no large conformational changes were required to accommodate the peptide. The central proline residue of the peptide, corresponding to Pro-101 of IscU, could be positioned into a hydrophobic pocket in the center of the cleft consistent with the key role in recognition and binding observed for this residue in biochemical studies. However, the peptide could also be modeled in the opposite direction in the cleft, and it was not possible to quantitatively distinguish between the predicted stability of the two orientations. The reverse binding orientation appeared to provide favorable electrostatic interactions that were not possible in the DnaK-like forward orientation suggesting that the reverse orientation might be favored.

The issue of whether HscA exhibits a specific directional preference for substrate binding was investigated in more detail by Tapley and Vickery (2004) using a fluorescence labeling and quenching strategy. HscA was selectively labeled on opposite sides of the SBD with the fluorescent probe bimane, and the ability of IscU-derived peptides having tryptophan at the N- or C-terminus to quench bimane fluorescence was measured. Quenching was found to be highly dependent on the position of tryptophan in the peptide and the location of bimane on HscA implying a strong directional preference for peptide binding. Differences in the quenching effectiveness of the N- and C-terminal labeled forms indicated that the peptide bound in the reverse direction relative to DnaK-peptide complexes. This was the first instance in which the reverse binding direction had been observed and established that substrate binding orientation can vary among different Hsp70 isoforms. Similar experiments with IscU revealed that the full-length protein binds in the same orientation as isolated peptides and that binding orientation is unaffected by the co-chaperone HscB. These findings place significant restrictions on the positioning of different regions of the IscU polypeptide relative to HscA as residues of IscU that are N- and C-terminal to the LPPVK binding region will be positioned on opposite sides of the SBD in the HscA-IscU complex.

HscA Crystallographic Studies

The crystal structure of a SBD fragment of *E. coli* HscA bound to an IscU-derived peptide (^{98}ELPPVKIHC^{106}) was determined to a resolution of 1.95 Å by Cupp-Vickery *et al.* (2004). The overall structure of the HscA(SBD)-ELPPVKIHC complex (Figure 6) is similar to that of the DnaK(SBD)-NRLLLTG complex (Zhu *et al.*, 1996). The peptide is bound in an extended conformation in a hydrophobic cleft within the β-subdomain, and a combination of nonpolar and hydrogen bonding interactions appear to contribute to the binding affinity and specificity of the interaction. The B-helix of the α-subdomain lies immediately above the binding cleft but does not make direct contact with the peptide and thus does not appear to play a direct role in determining substrate specificity. The central proline residue of the bound peptide is completely buried and is held in a hydrophobic pocket in the middle of the cleft (designated position "0"). The extensive interactions between this proline and residues forming the pocket are in accord with the key function for Pro-101 of IscU indicated by mutagenesis studies (Hoff *et al.*, 2003). Charged residues at each end of the peptide make electrostatic interactions with residues on the surface of the β-subdomain. The orientation of the peptide in the crystal complex is consistent with the reverse binding direction predicted by solution

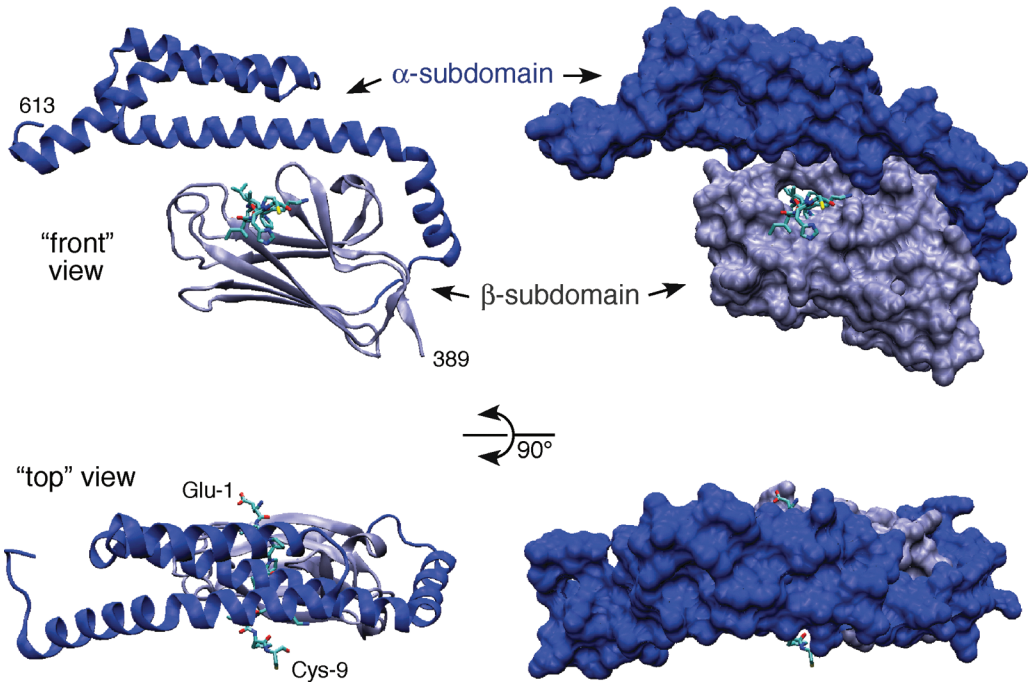

FIGURE 6 **Crystal structure of an HscA(SBD)-peptide complex. Ribbon and surface representations of the HscA substrate binding domain are shown with the α-subdomain blue and the β-subdomain gray. Bound peptide (ELPPVKIHC) is colored by atom type (carbon, *cyan*; nitrogen, *blue*; oxygen, *red*). PDB coordinate file 1u00 (Cupp-Vickery *et al.*, 2004).**

fluorescence studies with the N-terminus on the "back" side and C-terminus on the "front" side of the SBD (Tapley and Vickery, 2004). Assuming that the IscU protein binds to full-length HscA in a similar manner the N- and C-terminal regions of IscU will be separated from one another by the width of the β-subdomain (>16 Å). Complex formation would thus be expected to have pronounced effects on IscU structure and its ability to function as a [FeS]-cluster scaffold.

The peptide in the HscA(SBD)-ELPPVKIHC complex appears to be trapped in place by the "lid-like" α-subdomain, but the mechanisms involved in IscU binding and release are not well understood. A threading mechanism is viewed as implausible for Hsp70s and conformational changes involving the α-subdomain are considered likely to be required (Zhu *et al.*, 1996). Such conformational changes would be subject to allosteric regulation and dependent upon the nucleotide-bound state of the chaperone. The rates of binding and release of IscU are faster for the HscA·ATP T-state complex than for the HscA·ADP R-state complex (Silberg *et al.*, 2004) suggesting that the structure may somehow "open" to allow facilitate substrate exchange following ATP binding and T-state formation.

The structure of a complete form HscA is needed to fully understand the molecular basis of allosteric regulation of IscU interactions. Aoto *et al.* (2005) succeeded in obtaining crystals of a partially truncated form containing both nucleotide and substrate binding domains and bound to the IscU-derived peptide WELPPVKI. The HscA protein was shortened by deleting a 17-residue N-terminal extension not found in other Hsp70s and the α-subdomain of the SBD which is not required for IscU binding or allosteric cooperativity (Silberg *et al.*, 2001). Crystals suitable for x-ray diffraction analysis could be prepared in the absence of nucleotide, and recent analysis of diffraction data obtained with these crystals has allowed refinement of a structural model to 2.9Å resolution (Cupp-Vickery *et al.*[1]) (Figure 7). The structure of the truncated β-subdomain-SBD-peptide complex is very similar (*r.m.s.d.* 1.0Å) to that observed

[1]Cupp-Vickery, A.R., Aoto, P., and Vickery, L. E. 2007 (manuscript in preparation).

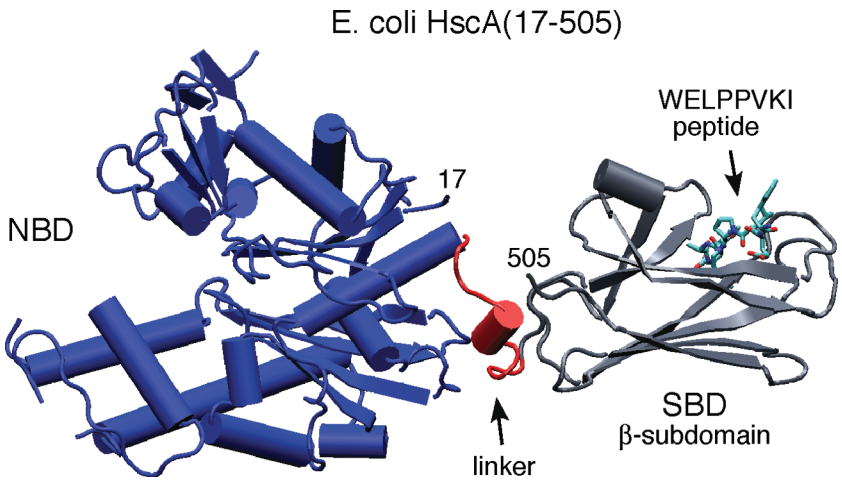

FIGURE 7 **Crystal structure of a HscA (17–505). A truncated form of HscA lacking N-terminal residues 1 to 16 and the α -subdomain of the SBD (residues 506 to 616) was crystallized in the presence of the IscU-derived peptide WELLPVKI and in the absence of nucleotide. Secondary structure elements of the NBD (residues 17–378; *blue*), linker region (residues 379–390; *red*), and SBD (residues 391–505; *gray*) are indicated: α-helices, cylinders; β-strands, *arrows*; loops, *ropes*. The peptide is shown as a stick structure and is colored by atom type (carbon, *green*; nitrogen, *blue*; oxygen, *red*).**

in the complete SBD-peptide complex (Cupp-Vickery *et al.*, 2004) indicating that removal of the α-helical lid has little effect on the peptide binding region. As with other Hsp70s the NBD consists of two major domains that form a deep nucleotide binding cleft. The cleft has an "open" structure resembling Hsp70-nucleotide exchange factor complexes such as GrpE-bound DnaK(NBD) (Harrison *et al.*, 1997) and Bag-bound Hsc70(NBD) (Sondermann *et al.*, 2001). This open structure is consistent with the fast intrinsic nucleotide exchange rates observed for HscA (Silberg and Vickery, 2000). The most surprising aspect of the structure is that the NBD and the β-subdomain of the SBD have no direct interactions with one another apart from the short linker segment connecting the domains. Modeling of the α-subdomain of the SBD (Cupp-Vickery *et al.*, 2004) into the HscA (17–505) crystal structure provided few additional contact sites near the linker region. Assuming that the observed structure reflects that of the R-state (nucleotide free from or ADP-complex) in solution, the lack of substantial interdomain contacts suggests that allosteric communication must be mediated by interactions unique to the T-state ATP-complex.

The crystal structure of *E. coli* HscA (17–505) differs in important aspects from structures proposed for other Hsp70s, including an NMR model of a similarly truncated form of DnaK and the crystal structure of an Hsc70 mutant. The exact positions of the NBD and the SBD observed in the HscA (17–505) crystal differ from those of a model based on solution NMR studies of a truncated form of DnaK from *Thermus thermophilus* (Revington *et al.*, 2005). In that study residual dipolar couplings were used with chemical shift measurements to determine the relative orientations of the NBD and SBD and to generate an approximate structural model. Although the exact positions of the NBD and SBD and the structure of the linker region could not be determined, the $DnaK_{Tth}$ solution model suggested that the two domains have different orientations compared to HscA (17–505). The HscA (17–505) structure differs even more significantly from the crystal structure observed for a mutant form of bovine Hsc70 (Jiang *et al.*, 2005). In the Hsc70 structure the NBD and SBD have extensive contacts that include a large number of interactions of the A-helix of the α-subdomain of the SBD with the NBD. A concern with the Hsc70 structure, however, arises from the mutations employed to obtain the crystals. Charged residues present in the wild-type protein that were replaced with alanine in the mutant were found to be localized within the observed NBD-SBD interface. The failure of wild-type Hsc70 to yield this crystal form suggests that the wild-type residues may cause unfavorable interactions in the conformation observed in the crystals. Moreover, steric constraints imposed by packing interactions between the domains in the observed crystal structure preclude positioning of the side chain of the wild-type residues within the model. This raises the question of whether a conformation similar to that observed in the crystal can

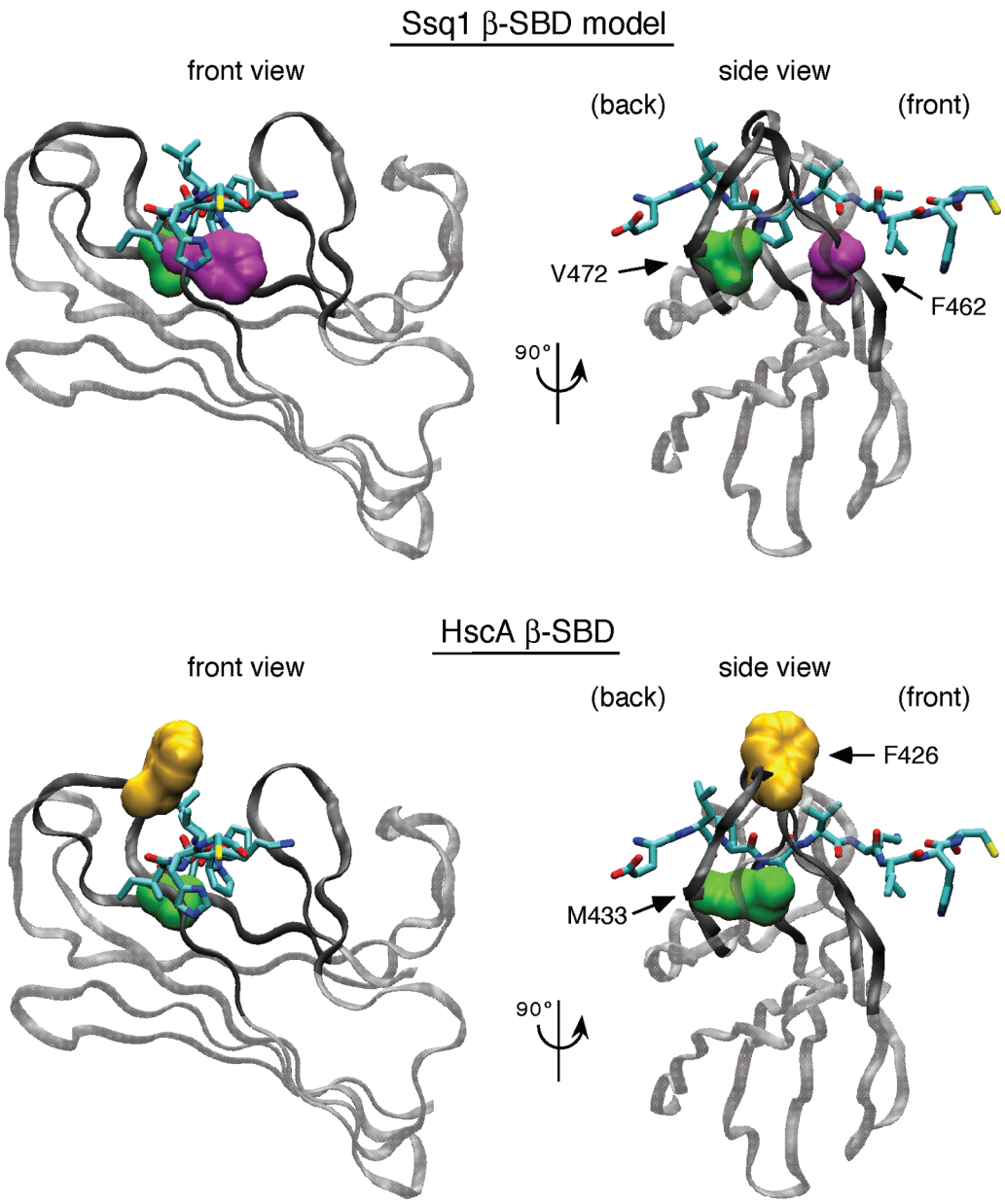

FIGURE 8 **Peptide binding clefts of Ssq1 and HscA. The *β*-subdomain of the SBDs are shown as ribbons with the darker segment representing backbone regions comprising the substrate cleft. The front view is the same as that shown in Figure 6. Selected side chains of Ssq1 and HscA are shown as surface representations, and Isu/IscU-derived peptides are shown as stick models (carbon, *cyan*; nitrogen, *blue*; oxygen, *red*). The Ssq1 structure was generated by homology modeling using the HscA(SBD)-ELPPVKIHC complex structure, PDB file 1u00 (Cupp-Vickery *et al.*, 2004).**

occur in the wild-type protein and whether the structure observed in the crystal is functionally relevant. Additional structural studies on different forms of HscA and of other Hsp70s will be required to obtain a better understanding of chaperone action and regulation.

Determinants of Substrate Recognition

Structural features of the peptide binding region that contribute to chaperone substrate recognition have been studied for both Ssq1 and HscA. Craig, Marszalek and coworkers (Knieszner *et al.*, 2005) investigated the effects of substitutions within the peptide binding cleft of yeast Ssq1 on interactions with the scaffold protein Isu and with Isu-derived peptides (*cf.* Figure 8). *In vitro* biochemical measurements were used to determine binding affinity, and growth phenotypes were used to assess *in vivo* consequences. Replacement of Phe-462 with serine abolished the ability of Ssq1 to interact with Isu-like peptides and resulted in a growth phenotype, mitochondrial iron accumulation

and iron-sulfur enzyme activities similar to Δ*ssq1* cells. These findings establish the importance of Phe-462, conserved among Hsp70s, for chaperone function and underscore the importance of Ssq1-Isu1 interactions *in vivo*. Replacement of Val-472 with phenylalanine reduced affinity for Isu and Isu-like peptides and the allosteric coupling between Isu binding and enhancement of ATPase activity *in vitro*, but caused only moderate changes *in vivo* (growth, mitochondrial iron accumulation and iron-sulfur enzyme activities). *In vitro* studies further showed that high concentrations of Isu1 and Jac1 could partially compensate for the low substrate affinity of Ssq1(V472 F), and *in vivo* studies revealed that the *ssq1(V472)* cells had elevated levels of Isu1. The restoration of phenotypic properties and the coupling of regulation of Isu1 levels to Ssq1 activity provide strong evidence for the role of Ssq1-Isu1 interactions in iron-sulfur protein formation.

The specificity of different regions of the peptide binding cleft of *E. coli* HscA was investigated by Tapley *et al.* (2006). A cellulose-based peptide array was used to individually replace each amino acid within a peptide corresponding to ^{98}ELPPVKI^{104} of IscU and to determine qualitative effects on HscA binding affinity. HscA was found to be able to recognize peptides having substitutions at all sites except for cleft position 0 where proline is required and cleft position -2 (front side) where a basic residue, lysine or arginine, is required. These results are consistent with earlier biochemical studies establishing the importance of Pro-101 and Lys-103 in IscU for high affinity binding. They also agree with the crystal structure of HscA(SBD)-ELPPVKIHC in which cleft position 0 corresponds to the central hydrophobic pocket where proline was observed to bind, and cleft position -2 corresponds to where lysine was observed to bind and interact with Glu-406 (Cupp-Vickery *et al.*, 2004). Because of the key role of cleft position 0 substitutions were made in one of the residues forming the "arch" above the pocket, HscA(F426 A), and in the residue forming the base of the pocket, HscA(M433V) (Figure 8). Reducing the size of the sidechain of arch residue 426 did not significantly affect peptide binding preference but resulted in a general decrease in peptide binding affinity. Kinetic studies revealed that the decrease in affinity was due to increased dissociation rates consistent with a role for Phe-426 in stabilizing the substrate complex. Replacement of Met-433 with valine, on the other hand, reduced the binding affinity for IscU-like peptides and altered the specificity in favor of a peptide containing leucine rather than proline in the central position. These results suggest that the SBD cleft structure is an important determinant of substrate selectivity.

CHAPERONES AND IRON-SULFUR CLUSTER TRANSFER

Genetic and biochemical studies have provided compelling evidence for the importance of chaperone-scaffold interactions in iron-sulfur protein maturation, but the exact roles of the chaperones remain to be determined. Chaperones could be involved in regulating the formation of [FeS]-clusters on the scaffold proteins, the transfer of clusters from scaffolds to apo-acceptor proteins, and/or the type of cluster formed or transferred. Several lines of evidence, including *in vivo* studies of yeast Ssq1/Jac1/Isu1 systems and *in vitro* studies of bacterial HscA/HscB/IscU systems, suggest that the main function of the chaperones is most likely coupled to the transfer of [FeS]-clusters subsequent to their formation. In studies with *S. cerevisiae* Mühlenhoff *et al.* (2003) genetically controlled the expression of Ssq1 and Jac1 and found that depletion of either chaperone or co-chaperone resulted in accumulation of iron on Isu1 and a reduction in the amount of iron present in iron-sulfur enzymes. Thus Ssq1 and Jac1 do not appear to be necessary for cluster assembly, but are both required for efficient cluster transfer. Dutkiewicz *et al.* (2006) further tested this hypothesis using Isu1 mutants having altered Ssq1 recognition sequences that resulted in poor binding to the chaperone. Strains expressing Isu1(P134S) or Isu1(K136S) were able to incorporate iron into the mutant scaffold proteins, but iron levels in the iron-sulfur enzyme aconitase were greatly reduced. These findings underscore the importance of chaperone-scaffold interactions for iron-sulfur protein maturation and provide additional evidence for a primary role of the chaperones in cluster transfer.

In vitro studies of the effects of chaperone-scaffold interactions on [FeS]-cluster formation and transfer were first described by Cowan and coworkers (Wu *et al.*, 2005) for components from the thermophilic bacterium, *Thermotoga maritima*. Because *T. maritima* does not have a specific HscA homolog the general *T. maritima* chaperone DnaK was employed in these studies. DnaK was found to modestly stabilize the IscU[FeS] complex, and to slightly *inhibit* cluster transfer from IscU[FeS] to the ferredoxin. These results

suggested that bacterial systems might behave differently from the yeast mitochondrial system where results suggested that the chaperones are necessary for cluster transfer. The effects observed with the *T. maritima* system, however, required high concentrations of DnaK in excess of IscU levels and were found to be independent of ADP, ATP, and DnaJ; in addition, the nucleotide exchange protein GrpE was not included. The high concentrations required and the lack of nucleotide and co-chaperone effects raise questions about the physiological relevance of the findings.

Recent *in vitro* studies on the effects of HscA and HscB on IscU cluster formation and transfer in other bacteria have yielded results more in accord with the *in vivo* studies of the yeast Ssq1/Jac1/Isu1 system. Bonomi *et al.* (2005) found the *E. coli* HscA destabilized the IscU[FeS] complex (evidenced by cluster spectral changes and iron release to chelators) and also enhanced the rate of cluster transfer from IscU[FeS] to apo-ferredoxin. These effects required HscB and ATP as expected for physiological chaperone activities. Stimulation of the rate of cluster transfer was directly proportional to the concentration of HscA and HscB and could be observed at chaperone:IscU ratios as low as 0.1:1 consistent with a catalytic role for the chaperones. Chandramouli and Johnson (2006) carried out similar studies with the HscA/HscB/IscU system from *Azotobacter vinelandii*. HscA together with HscB and ATP enhanced cluster transfer from holo-IscU to apo-ferredoxin and gave maximal cluster transfer rates corresponding to a 30-fold rate enhancement compared to uncatalyzed transfer. A kinetic model for cluster transfer from IscU[FeS] to apo-ferredoxin was proposed based on the ATPase reaction cycle (Silberg *et al.*, 2004). It was not possible, however, to determine whether transfer occurred from the T-state HscA·ATP-IscU[FeS] complex or the R-state HscA·ADP-IscU[FeS] complex (*cf*. Figure 4). Recent studies by Bonomi et al.[2] suggest that formation of the R-state HscA·ADP-IscU[FeS] complex is required for catalysis of cluster transfer. An HscA mutant lacking ATPase activity but capable of T-state formation, HscA(T212V), was found to be inactive in cluster transfer assays. This finding suggests that ATP hydrolysis and subsequent R-state formation are required for cluster activation and catalysis of transfer. However, it remains unknown whether cluster transfer occurs while IscU[FeS] is bound to HscA or occurs only after release of the [FeS]-scaffold complex from the chaperone.

[2]Bonomi, F., Iametti, S., Ta, D. T., and Vickery, L.E. 2007 (manuscript in preparation).

RÉSUMÉ AND FUTURE DIRECTIONS

The presence of specialized chaperone systems for iron-sulfur protein formation in eubacteria and yeast has proved to be of great value in studies of iron-sulfur protein biogenesis. The bacterial systems have facilitated identification and biochemical characterization of the components involved, and the yeast systems have provided genetic means of evaluating function and establishing *in vivo* significance. The general biochemical properties of the bacterial (HscA/HscB) and yeast (Ssq1/Jac1) chaperones have been well defined, and structures of the chaperones are beginning to provide insight into their activities and the molecular basis of their interactions with the IscU and Isu scaffold proteins. Additional structural studies, especially characterization of the various protein complexes and the different conformational states of the chaperones, are needed to provide a better understanding of the nature of the interactions and how these regulate chaperone activity.

Current evidence suggests that the primary role of the chaperones is likely to facilitate cluster release to apo-acceptor proteins, but the exact nature of the effects on the [FeS]-scaffold complexes and how the chaperones bring about these changes remain unknown. In fact, the reason a chaperone system is required is not well understood. It may be that [FeS]-scaffold complexes must be sufficiently stable to prevent spontaneous degradation and/or oxidative damage, and chaperones may provide a means to transiently destabilize the complex in order to allow cluster release to acceptor proteins. Involvement of chaperones in cluster transfer may also provide a means to control iron-sulfur protein formation, and additional studies on chaperone-scaffold interactions and cluster release may provide insights into regulatory mechanisms. Furthermore, it should be noted that *in vivo* studies suggest that chaperones are necessary for formation of both [2Fe2S]- and [4Fe4S]-proteins, while *in vitro* biochemical studies have only been carried out using [2Fe2S]-complexes of IscU. Studies are needed to determine whether the chaperones have specific effects on different classes of [FeS]-clusters and whether

the chaperones play any type of regulatory role in determining the types of holo-proteins formed.

It will also be important to extend studies to the chaperones involved in iron-sulfur protein biogenesis in higher eukaryotes. Current evidence suggests that most organisms likely employ a multifunctional mtHsp70 that functions with specialized co-chaperones, but the general properties of the chaperones and details of their interactions with scaffold proteins remain to be determined. Finally, it is also possible that there may be additional factors that interact with the chaperones and or scaffold proteins during the cluster assembly and/or transfer processes. Identification of these components and characterization of their effects on the process may provide new insight into the underlying mechanisms involved as well as how the iron-sulfur protein biosynthesis is regulated.

ACKNOWLEDGMENT

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