The Fe/S Assembly Protein IscU Behaves as a Substrate for the Molecular Chaperone Hsc66 from Escherichia coli*

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IscU, a NiFU-like Fe/S-escort protein, binds to and stimulates the ATPase activity of Hsc66, a hsp70-type molecular chaperone. We present evidence that stimulation arises from interactions of IscU with the substrate-binding site of Hsc66. IscU inhibited the ability of Hsc66 to suppress the aggregation of the denatured model substrate proteins rhodanese and citrate synthase, and calorimetric and surface plasmon resonance measurements showed that ATP destabilizes Hsc66-IscU complexes in a manner expected for hsp70-substrate complexes. Studies on the interaction of IscU with Hsc66 truncation mutants further showed that IscU does not bind the isolated ATPase domain of Hsc66 but does bind and stimulate a mutant containing the ATPase domain and substrate binding β-sandwich subdomain. These results support a role for IscU as a substrate for Hsc66 and suggest a specialized function for Hsc66 in the assembly, stabilization, or transfer of Fe/S clusters formed on IscU.

Experimental Procedures

Materials—Escherichia coli DH5αFIQ cells were from Life Technologies, Inc. Enzymes for DNA manipulation were obtained from Roche Molecular Biochemicals, New England Biolabs, Inc., or U. S. Biochemical Corp. Synthetic nucleotides were obtained from Genosys. Bacterial growth media components were from Difco, and other reagents were from Sigma.

Overexpression and Purification of Proteins—Recombinant Hsc66, Hsc20, and IscU were expressed and purified as described previously (20, 21). Vectors for overexpressing truncated forms of Hsc66, pTrc66(D383stop) for residues 2–382, and pTrc66(D506stop) for residues 2–505, were made by introducing stop codons into the vector encoding Hsc66 (pTrc66; see Ref. 20) using the Unique-Site Elimination method (CLONTECH). Both truncation mutants were overexpressed and purified using similar methods as reported for full-length Hsc66 (20).

ATPase Assays—Steady-state ATPase rates were determined at 23 °C in HKM buffer (50 mM Hepes, pH 7.3, 150 mM KCl, and 10 mM MgCl₂) containing 1 mM dithiothreitol (DTT) and 0.4 mM ATP as previously reported (18, 20, 21) by measuring phosphate released using a coupled enzyme assay with the EnzCheck phosphate assay kit (Molecular Probes).

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† Material and methods, including purification, are found in the published paper online at http://www.jbc.org.
Rhodanese and Citrate Synthase Aggregation Assays—The aggregation of bovine rhodanese and porcine citrate synthase were performed in HKM buffer containing 1 mM ADP as described previously (18).

Surface Plasmon Resonance (SPR) Analysis—SPR methods were carried out at 25 °C with a Biacore 3000 instrument (Piscataway, NJ) using methods described previously (21). Hsc66 in the presence of MgADP was randomly cross-linked to the surface of the sensor chip using amine coupling as recommended by the manufacturer. IscU was injected over the immobilized Hsc66 in HKM buffer containing 1 mM DTT and either 1 mM ADP or 1 mM ATP. Data are reported as changes in relative response units (RU). Similar results were obtained when Hsc66 was immobilized to the surface of a sensor chip in the presence of MgATP indicating that the differences in binding kinetics observed were not the result of the conditions of Hsc66 immobilization.

Isothermal Titration Calorimetry—A Microcal (Amherst, MA) Omega titration calorimeter was used to investigate the binding of IscU to Hsc66, Hsc:2–505, and Hsc:2–382 in HKM buffer containing 1 mM DTT and either 1 mM ADP or 1 mM ATP. The titration calorimeter was used to investigate the binding of IscU to Hsc66. Data are reported as changes in relative response units (RU). Similar results were obtained when Hsc66 was immobilized to the surface of a sensor chip in the presence of MgATP indicating that the differences in binding kinetics observed were not the result of the conditions of Hsc66 immobilization.

RESULTS

IscU Effects on Hsc66 Chaperone Activity—To investigate the possible role of IscU as a specific substrate for Hsc66, we examined the ability of IscU to compete with the model peptide substrates rhodanese and citrate synthase (18). Assays were carried out by measuring the effect of Hsc66/ADP on the extent of rhodanese or citrate synthase aggregation in the presence of varying levels of IscU. The effect of IscU on the ability of Hsc66 to suppress the aggregation of chemically denatured rhodanese is shown in Fig. 1A. IscU inhibited Hsc66 suppression of rhodanese aggregation in a concentration-dependent manner with a molar ratio of IscU to Hsc66 of 1:1 resulting in ~75% inhibition of Hsc66 chaperone activity (15 min), and a ratio of 5:1 giving >95% inhibition. IscU alone had no effect on rhodanese aggregation suggesting that the changes in turbidity caused by addition of IscU to reactions containing Hsc66 are not due to IscU directly enhancing rhodanese aggregation.

The effect of IscU on the ability of Hsc66 to suppress the aggregation of thermally denatured citrate synthase is shown in Fig. 1B. IscU inhibited Hsc66 suppression of citrate synthase aggregation, and this effect was dependent on the level of IscU with a molar ratio of IscU to Hsc66 of 2:5:1 inhibiting Hsc66 chaperone activity ~85% (40 min). IscU alone had no effect on citrate synthase aggregation. The competition of IscU with both rhodanese and citrate synthase for binding to Hsc66 is consistent with interaction of IscU with the peptide-binding domain of Hsc66 and suggests that IscU may form a stable complex with the high peptide affinity ADP state of Hsc66.

Nucleotide Effects on Hsc66 and IscU Binding—A hallmark of hsp70 proteins is their ability to modulate substrate binding in a nucleotide-dependent manner. The ADP complexes of hsp70 proteins display high affinity for peptide substrates and exhibit slow on and off rates, and exchange of ADP for ATP results in conformational changes leading to a low substrate affinity form with faster substrate association and dissociation rates (8–13). Because IscU was found to compete with model peptide substrates for binding to Hsc66, it was of interest to determine whether IscU binding to Hsc66 is regulated in a manner similar to that observed for other hsp70 substrates.

In initial experiments, SPR analysis was used to investigate nucleotide effects on the interaction of IscU with Hsc66. Fig. 2 shows the results of titrations in which Hsc66 was randomly cross-linked to the sensor chip and exposed to different concentrations of IscU in the presence of ADP or ATP. The extent of binding of IscU to Hsc66 was similar in the presence of either nucleotide, but the apparent affinity of Hsc66 for IscU was greater in the presence of ADP (K_D(ADP) = 9 μM) compared with that observed in the presence of ATP (K_D(ATP) = 37 μM). Examination of the sensorgrams (insets Fig. 2) also reveals differences in the binding kinetics as a function of nucleotide. IscU association in the presence of ATP is faster than that observed in the presence of ADP, and the half-time for IscU dissociation in the presence of ATP (t_1/2 = 2 s) is ~30-fold faster than that observed in the presence of ADP (t_1/2 = 60 s). These results indicate ATP destabilizes Hsc66-IscU complexes in a manner similar to that observed for other hsp70-substrate interactions (8–13) and are consistent with interaction of IscU with the peptide-binding site of Hsc66.

Because of possible complications in the SPR binding studies arising from surface and/or immobilization effects, we also used isothermal titration calorimetry (ITC) to more accurately quantify the interaction of IscU with Hsc66. Fig. 3 shows the enthalpic changes observed in an experiment in which succes-
binding sites, and KD to the data indicates the presence of a single, high affinity result of binding. Injection of IscU into a cell containing Hsc:z binds model peptide substrates, and a C-terminal helical cap but contained both the N-terminal ATPase domain and the peptide-binding C-terminal peptide-binding domain highly conserved N-terminal ATPase domain proteins are composed of two functionally distinct domains, a \( b \)-sandwich region that directly binds model peptide substrates, and a C-terminal helical cap that lies above the peptide binding pocket of the \( b \)-sandwich (25). To investigate which domain(s) of Hsc66 are required for IscU binding, we constructed two truncation mutants (Fig. 4, A and B). The first mutant, designated Hsc:2–505, lacked the C-terminal helical cap but contained both the N-terminal ATPase domain and the peptide-binding \( b \)-sandwich subdomain (residues 2–505). The second mutant, designated Hsc:2–382, contained only the ATPase domain (residues 2–382).

The effect of IscU on the ATPase activity of Hsc66, Hsc:2–505, and Hsc:2–382 was compared to characterize the region(s) of Hsc66 required for IscU binding (Fig. 4C). Both truncation mutants exhibited intrinsic ATPase activity that was slightly greater than that of full-length Hsc66. The ATPase activities of full-length Hsc66 and Hsc:2–505 were stimulated by IscU indicating the C-terminal helical cap is necessary for IscU binding and activation. The activity of the ATPase domain fragment Hsc:2–382, however, was not affected by IscU indicating that the peptide-binding \( b \)-sandwich subdomain is required for IscU stimulation of ATPase activity as expected if IscU is a substrate for Hsc66. To determine whether IscU interacts with the isolated ATPase domain Hsc:2–382 in a manner not manifested as effects on ATPase activity, we also used ITC to measure enthalpic changes that might arise as a result of binding. Injection of IscU into a cell containing Hsc:2–382 and ADP did not result in any detectable enthalpic changes suggesting that IscU does not interact directly with the isolated ATPase domain (data not shown).

Two approaches were used to further investigate the role of the C-terminal helical cap of Hsc66 in interactions with IscU. To investigate interactions of IscU with the ATP-bound state of Hsc:2–505, we examined the concentration dependence of IscU stimulation of Hsc:2–505 ATPase activity (see Fig. 5A). The concentration of IscU required for half-maximal stimulation of Hsc:2–505 activity \( (K_m \approx 31 \mu M) \) is similar to that previously reported for full-length Hsc66 \( (K_m \approx 34 \mu M); \) Ref. 21), although the maximal stimulation observed for Hsc:2–505 \((14\text{-fold})\) is slightly greater than that of full-length Hsc66 \((8\text{-fold}; \) Ref. 21). These findings indicate that removal of the C-terminal helical cap of Hsc66 has little effect on either the affinity of ATP-bound Hsc66 for IscU or its ability to couple IscU binding with increased ATPase activity.

To investigate interactions of IscU with the ADP-bound state of Hsc:2–505, we measured the thermodynamics of IscU binding to Hsc:2–505 in the presence of ADP. Fig. 5B shows the results of an ITC experiment in which successive additions of IscU were made to a cell containing the Hsc:2–505:ADP complex. The binding affinity observed \( (K_m \approx 26 \mu M) \) is \(-16\text{-fold} \) weaker than that of full-length Hsc66 under similar conditions \( (K_m \approx 1.6 \mu M; \) Fig. 3). Instead, it is similar to the apparent affinity observed for the ATP-bound state of Hsc:2–505 (31 \mu M; Fig. 5A). Thus, the C-terminal helical cap of Hsc66 is required for the increased binding affinity for IscU that occurs following ATP hydrolysis.

**DISCUSSION**

In earlier studies we found that both Hsc20 and IscU stimulate the ATPase activity of Hsc66 and regulate the rate of conversion of Hsc66 between its different peptide affinity states (20, 21). Whereas Hsc20 shares sequence similarities with DnaJ-type auxiliary co-chaperones and is thought to function in a manner similar to these proteins (18), IscU represents a novel ATPase stimulatory protein whose mechanism of action was unclear. IscU was also found to bind to Hsc20, and in the presence of Hsc20 the concentration of IscU required for half-maximal stimulation of Hsc66 ATPase activity was reduced (21). The interaction between IscU and Hsc20 resembles the interaction of peptide substrates with DnaJ, a hsp40 co-chaperone. DnaJ decreases the concentration of peptide substrates required for half-maximal stimulation of DnaK ATPase activity (26), and thereby serves to target these substrates to the high peptide affinity ADP state of DnaK (27). Similarities in the actions of Hsc20 and DnaJ suggested that the increased binding affinity of Hsc66 for IscU observed in the presence of Hsc20 could reflect the role of IscU as a substrate.

The results described herein provide direct evidence that IscU behaves as a substrate for Hsc66. IscU was shown to compete with model peptide substrates for binding to the Hsc66:ADP complex consistent with IscU binding to the high peptide affinity ADP state of Hsc66. Affinity sensor studies also showed that nucleotides regulate Hsc66 binding to IscU in a manner consistent with that observed for nucleotide effects on hsp70-peptide substrate interactions (8–13). ATP-bound Hsc66 exhibited faster association and dissociation rates for IscU compared with ADP-bound Hsc66 indicating that ATP destabilizes Hsc66-IscU complexes. In addition, calorimetric studies revealed that IscU binds to Hsc66 at a single site with an affinity \( (K_m \approx 1.6 \mu M) \) \(-20\text{-fold} \) greater than the concentration of IscU required for half-maximal stimulation of Hsc66 ATPase activity \( (K_m \approx 34 \mu M); \) Ref. 21). Assuming that the \( K_m \) observed in ATPase stimulation assays reflects the binding affinity of
A. Hsc66 Constructs

B. SDS-PAGE

C. ATPase Activities

\[ K_m = 31 \mu M, \quad K_D = 26 \mu M \]

**Fig. 4. Effect of IscU on the ATPase activity of Hsc66 truncation mutants.** A, diagrams of the domain composition of Hsc66 constructs. B, SDS-PAGE analysis of 10 µg each of purified Hsc66 (lane 1), Hsc2–505 (lane 2), Hsc2–382 (lane 3). C, steady-state ATPase activity of Hsc66, Hsc2–505, and Hsc2–382 were determined in HKM buffer containing 400 µM ATP at 23 °C in the absence (solid bars) and presence of 100 µM IscU (open bars).

**Fig. 5. Nucleotide effects on IscU and Hsc2–505 interactions.**

A, effect of IscU on the ATPase activity of Hsc2–505. Results are reported as the increase in basal ATPase rates at 23 °C. The curve shown represents a best fit to the data for a maximal stimulation of 13.8-fold and half-maximal stimulation at 31 µM. B, ITC analysis of IscU binding to Hsc2–505. A series of 35 equivalent 8-µl aliquots of 1.5 mM IscU were injected into a cell containing 1.348 ml of 100 µM Hsc2–505 at 25 °C in HKM buffer containing 1 mM DTT and 1 mM ADP. Integrated heats due to binding, \( Q_m \), are plotted versus the molar ratio of IscU to Hsc2–505 in the titration cell. The solid line represents a best-fit curve assuming 0.83-binding sites, \( K_p = 26 \mu M, \Delta H = 12.85 \) kcal/mol, and \( \Delta S = 64.1 \) e.u.

IscU for the ATP state of Hsc66, the differences in affinities of the ADP and ATP-bound states is similar to that observed with other hsp70-substrate complexes (9, 11, 12).

Studies examining the interaction of IscU with truncation mutants of Hsc66 provide further support that IscU behaves as a substrate for Hsc66. IscU stimulated the ATPase activity of a mutant containing the ATPase domain and the peptide-binding \( \beta \)-sandwich region (Hsc2–505), but no interactions were observed with a mutant composed solely of the ATPase domain (Hsc2–382). These results establish that the \( \beta \)-sandwich region of Hsc66 is essential for IscU binding as is the case for substrate binding to other hsp70 proteins (25).

Although the Hsc66 C-terminal helical cap was not required for IscU stimulation of Hsc66 ATPase activity, this subdomain was required for coupling of ATP hydrolysis with conformational changes that increase Hsc66 binding affinity for IscU. In contrast to full-length Hsc66 in which Hsc66-ADP complexes exhibit ~20-fold higher affinity for IscU compared with ATP complexes, ADP and ATP complexes of Hsc2–505 displayed similar IscU affinities indicating that both the \( \beta \)-sandwich and helical cap subdomains are required for high affinity binding. The C-terminal helical cap does not, however, appear to be required for all hsp70-substrate interactions. A recent report showed that DnaK mutants lacking the cap retain efficient coupling of ATP hydrolysis with increased substrate binding affinity (28). This may reflect structural differences between Hsc66 and DnaK or may arise from differences in the types of substrates recognized. The short peptide used in the DnaK studies (28) would not be expected to make extensive contacts with the C-terminal helical cap (25). The larger IscU protein, in contrast, may interact with both the \( \beta \)-sandwich and helical cap subdomains of Hsc66, and interactions of IscU with both subdomains may be required for high affinity binding. There is evidence that the helical cap may be important for regulatory interactions of the eukaryotic homologs of Hsc66. Mutations in the corresponding C-terminal subdomain of the yeast mitochondrial homolog of Hsc66, Ssq1, gives rise to phenotypic effects indicative of reduced chaperone activity (19).

The finding that IscU behaves as a substrate for Hsc66 raises the question of whether the Hsc66/Hsc20 chaperone system has evolved specifically to interact with IscU in Fe/S-cluster assembly or whether it serves a more general chaperone function in the cell. Like other hsp70 chaperones Hsc66 is able to bind denatured proteins (e.g. rhodanese and citrate synthase) and to prevent their aggregation (18). These “model substrates,” however, have no effect on the ATPase activity of Hsc66. IscU binding, in contrast, is coupled to the nucleotide state of Hsc66 and regulates the chaperone’s ATPase reaction cycle. IscU also exhibits specific interactions with the co-chaperone Hsc20 that is not observed with other proteins or peptides (18, 21). These results, together with genetic findings implicating yeast homologs of Hsc66, Hsc20, and IscU in iron-sulfur protein biogenesis (19), suggest that the primary cellular function of the Hsc66/Hsc20 chaperone system may be to interact with IscU in Fe/S-cluster generation.

While the studies described herein indicate that IscU binds as a substrate for Hsc66, the specific structural features of IscU required for interaction with Hsc66 are not known. Hsc66 may recognize a structured motif within IscU or may bind an unstructured region of IscU in an extended conformation similar to that recognized by DnaK (25). Further studies are also needed to investigate how Hsc66 and Hsc20 affect the function of IscU in iron-sulfur cluster assembly. The exact role the Hsc66/Hsc20 chaperone system plays in iron-sulfur cluster biogenesis remains unclear. The chaperone system may function

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\(^2\) The \( K_m \) obtained in ATPase assays is only a good estimate of the equilibrium dissociation constant of ATP-bound Hsc66 for IscU if binding of IscU is rapid relative to ATP hydrolysis (29).
in assembly or stabilization of Fe/S clusters on IscU or may facilitate transfer of Fe/S clusters formed on IscU to an apo-acceptor protein.

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