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(S) state, and that interaction with HscB shifts the D \(\rightleftharpoons\) S equilibrium toward the S-state (16). The structure of the S-state resembles that adopted by IscU when it contains either a \([2\text{Fe}-2\text{S}]\) cluster (17) or Zn\(^{2+}\)/H\(_{11001}\) ion (18), whereas the D-state appears not to interact with metal ions (19). We report here that the addition of a substoichiometric quantity of HscA decreases the D \(\rightleftharpoons\) S rate while not affecting the S \(\rightleftharpoons\) D rate, thus shifting the D \(\rightleftharpoons\) S equilibrium toward the D-state. In the presence of excess HscA or the HscA-ADP complex, IscU becomes transformed to a D-like state. By contrast, a variant of IscU that exists predominantly in the S-state (N90A) and the IscU-Zn\(^{2+}\)/H\(_{11001}\) complex that is fully in the S-state (19) are each only partially converted to the D-like state upon the addition of HscA. These results suggest that HscA, alone or as the ADP complex, binds to and stabilizes the D-state of IscU. The addition of ATP to the IscU-HscA complex causes release of IscU, which then resumes the D \(\rightleftharpoons\) S equilibrium. We propose a mechanism (Fig. 1) consistent with these findings in which cluster transfer from IscU is coupled to hydrolysis of HscA-bound ATP and the concomitant conformational change that enables HscA-ADP to bind and stabilize the D-state of IscU, which promotes both the release of the Fe-S cluster to an acceptor protein and the release of HscB.

**EXPERIMENTAL PROCEDURES**

Production and Purification of Proteins—HscB, [U-\(^{15}\text{N}\)]apo-IscU, and [U-\(^{13}\text{C},^{15}\text{N}\)]apo-IscU were prepared as described previously (11, 16). [U-\(^{15}\text{N},\text{frac-}^{2}\text{H}\)]Apo-IscU (where frac-\(^2\text{H}\) indicates fractionally \(^2\text{H}\)-labeled) was produced as follows. First, a single colony of transformed BL21 competent cells (Novagen) was transferred to 5 ml of LB medium containing 100 \(\mu\)g/ml ampicillin and incubated for \(\approx 8\) h at 37 °C. From this, a 0.1-ml inoculum was transferred to 5 ml of M9 minimal medium supplemented with 100 \(\mu\)g/ml ampicillin, 1 g/liter \(^{15}\text{NH}_4\text{Cl}\), and 5 g/liter glucose. The cells were adapted to growth in D\(_{2}\)O by stepwise growths of 0.1-ml inoculants in 5 ml of M9 medium dissolved in 20, 50, 80, and 100% D\(_{2}\)O, respectively; each cell growth was incubated for 8–12 h at 37 °C. A 0.1-ml inoculant from 5 ml of 100% D\(_{2}\)O/M9 medium was transferred to 50 ml of 100% D\(_{2}\)O/M9 medium, which was incubated for \(\approx 20\) h at 37 °C. The cell pellet harvested from 50 ml of medium was finally transferred to 500 ml of 100% D\(_{2}\)O/M9 medium supplemented with 100 \(\mu\)g/ml ampicillin, 1 g/liter \(^{15}\text{NH}_4\text{Cl}\), and 5 g/liter glucose. Overexpression was induced by the addition of 0.4 mM isopropyl \(\beta\)-D-thiogalactopyranoside at \(A_{600} = 0.8\), followed by further incubation for 6–8 h at 37 °C. This procedure, which leads to the deuteration of most H\(^{1}\) and H\(^{\text{d}}\) positions, results in significant increases in the intensities of \(^{1}\text{H}\)N signals from larger macromolecules (20). Harvested cell pellets were stored at \(-80\) °C until used.

The expression plasmids for full-length WT HscA and the HscA SBD (residues 389–616) were generous gifts from Dr. Larry E. Vickery (University of California, Irvine). The expression plasmid for HscA(T212V) was created by applying the QuikChange II site-directed mutagenesis kit (Stratagene) to the WT HscA expression vector. Full-length HscA and the HscA SBD were produced by procedures described previously (21, 22).
HscA and HscB Bind Different Conformational States of IscU

Full-length HscA and the HscA SBD were purified by procedures described previously (21, 22), except for the use of anion-exchange chromatography and size-exclusion chromatography instead of reversed-phase chromatography. For the additional anion-exchange chromatography step, a DEAE Bio-Gel column (Bio-Rad) was used with a 0–0.15 m NaCl gradient. Size-exclusion chromatography was accomplished with a HiLoad 16/60 Superdex 200 column (GE Healthcare) for full-length HscA or with a HiLoad 16/60 Superdex 75 column (GE Healthcare) for the HscA SBD. The elution buffer for this step consisted of 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.5 mM EDTA, and 150 mM NaCl.

NMR Spectroscopy—The solvent used for NMR samples contained either 50 mM HEPES/NaOH (pH 7.3), 10 mM MgCl₂, 150 mM KCl, and 5 mM DTT (HMKD buffer) or 50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, and 5 mM DTT (TED buffer) with 7% D₂O, 0.7 mM 2,2-dimethyl-2-silapentane-5-sulfonate, and 0.02% sodium azide. All NMR spectra were acquired on 600- or 900-MHz Varian VNMRS spectrometers equipped with a z-gradient cryogenic probe. Sample temperatures were maintained at 25 °C. NMRPipe (23) was used to process the raw NMR data; SPARKY (24) was used for data analysis; and the Newton software package (25) was used to determine peak volumes and their ratios.

The titration experiments of IscU and IscU(N90A) were initiated with 0.3 mM [U-¹⁵N]apo-IscU samples in HMKD buffer, and these samples were subsequently mixed with 1 or 2 eq of unlabeled full-length HscA or the HscA SBD. A two-dimensional ¹⁵N transverse relaxation optimized spectroscopy-heteronuclear single quantum correlation (TROSY-HSQC) spectrum linear as the result of relaxation effects. Previous NMR peaks were observed with mixing times of 0 and 20 ms, whereas substoichiometric amount (0.09 mM) of added HscA. Two-dimensional NMR data; SPARKY (24) was used for data analysis; and the Newton software package (25) was used to determine peak volumes and their ratios.

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RESULTS

HscA and HscB Bind Different Conformational States of IscU

To determine whether HscA interacts preferentially with the S- or D-state of IscU, we used two-dimensional ¹H-¹⁵N exchange spectroscopy to compare the S → D and D → S conformational exchange rates of [U-¹⁵N]apo-IscU in the absence and presence of substoichiometric HscA. In this experiment, the exchange peak horizontal to the D diagonal peak reports on the S → D reaction, and the exchange peak horizontal to the D diagonal peak reports on the D → S reaction (Fig. 2A). We measured the ratio of the cross-peak to the diagonal as a function of the mixing time of the experiment and determined the rate from the initial slope before exchange became obscured by relaxation effects (Fig. 2B) (26). We found that the addition of 0.25 eq of HscA reduced the D → S rate by a factor of ~2 (Fig. 2B, lower panels), whereas the S → D rate was unaffected within experimental error (Fig. 2B, upper panels). The addition of ADP did not induce further change in the exchange rate of IscU (data not shown). This result suggests that HscA preferentially interacts with and stabilizes the D-state of apo-IscU.

We used two-dimensional ¹⁵N TROSY-HSQC spectroscopy to follow the titration of a sample of [U-¹⁵N]apo-IscU with increasing amounts of unlabeled HscA. Before the addition of HscA, the spectrum (Fig. 3A) exhibited sets of peaks corresponding to those assigned to the S- and D-states of IscU (16, 19). Upon the addition of HscA, the peaks corresponding to the S-state decreased progressively and had nearly disappeared at a level of 2 eq of unlabeled HscA/eq of ¹⁵N-labeled IscU; only peaks corresponding to a D-like state remained (Fig. 3B). The subsequent addition of 2 eq of unlabeled IscU led to partial recovery of signals corresponding to the S-state (Fig. 3C) as the result of displacing some of the ¹⁵N-labeled IscU from the IscU-HscA complex; this result shows that the IscU-HscA interaction is reversible and that the observed effects were not the result of an artifact such as irreversible protein denaturation or proteolysis. The side chain ¹H-¹⁵N of Trp-76 (the only tryptophan residue in IscU) exhibited separate two-dimensional assignments to residues of apo-IscU in the S- and D-states (19) were used in obtaining residue-specific exchange rates. The experiments were repeated twice with two independently prepared samples to estimate errors from S.D.

Determination of the conformational states of apo-IscU under various conditions was accomplished by monitoring the volumes of the Lys-128 ¹H-¹⁵N peaks: δ₃, 7.76 ppm and δ₄, 126.2 ppm in the S-state and δ₃, 7.85 ppm and δ₄, 126.2 ppm in the D-state. Measurements were initiated with samples of 0.3 mM [U-¹⁵N]apo-IscU in HMKD buffer. To produce the complexes, we added 0.3 mM HscB, 0.3 mM HscA, or 0.3 mM HscA(T212V). We subsequently added 10 mM ADP or 10 mM ATP. The intensities of the Lys-128 S- and D-state ¹H-¹⁵N peaks were measured from two-dimensional ¹⁵N TROSY-HSQC spectra of each mixture. Nucleotide exchange on HscA has been shown to be fast and not to require an exchange factor (27). As a model for HscA bound to ATP, we used the HscA(T212V)-ATP complex because this variant exhibits negligible (if any) ATPase activity, yet it undergoes the nucleotide-induced conformational transition (28–30).
HscA and HscB Bind Different Conformational States of IscU

We were able to assign a large number of backbone signals in spectra of the IscU-HscA complex by collecting three-dimensional HNCO spectra of [U-13C,15N]apo-IscU as a function of residue number. The chemical shift differences were all $\leq 0.025$ ppm with the exception of the 0.04 ppm shift of Glu-95, a residue that flanks the L99PPVK103 motif (Fig. 4B). Signals corresponding to Val-73–Val-77, Gly-79, Glu-96, Val-102, Ile-108, Ala-110, Asp-112, Ala-117, and Lys-122 (indicated by red $\times$ in Fig. 4B) broadened as HscA was added and were no longer detected after the addition of a stoichiometric amount of HscA. The broadening likely arises from residues becoming immobilized by binding to HscA, and this conclusion is reinforced by the fact that several of the broadened signals correspond to residues flanking the L99PPVK103 motif of IscU, which is known to interact with HscA (8, 9, 22). Chemical shift analysis of the assigned signals from IscU complexed with HscA showed that the protein lacks secondary structure (Fig. 4C).

From these results, we conclude that the conformation of IscU bound to HscA closely resembles that of the D-state of free apo-IscU, which is dynamically disordered and exhibits no secondary structural elements (19). To illustrate this, on the three-dimensional structure of the S-state of IscU (Fig. 4D), residues of IscU shown to be disordered in the IscU-HscA complex are colored green, and residues whose signals broadened in the IscU-HscA complex are colored red; residues shaded black are ones whose signals were not observed in the spectrum of free IscU.

Because the S-state of apo-IscU is known to be stabilized by the N90A substitution (19) or by the addition of Zn$^{2+}$ (16, 19), we investigated the effects of adding HscA on the conformations of these two proteins. In comparison with wild-type IscU, much more of the S-state remained after the addition of 2 eq of HscA to [U-15N]apo-IscU(N90A) or to [U-15N]IscU-Zn$^{2+}$ (data not shown). These results indicate that HscA does not...
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**FIGURE 4.** Changes in the two-dimensional $^1$H-$^{15}$N spectrum of IscU upon the addition of HscA. A, representative examples of D-state signals from [U-$^{15}$N]$^2$H]-apo-IscU that are perturbed upon the addition of HscA. Shown is a two-dimensional $^1$H-$^{13}$C slice through a three-dimensional HNCO spectrum at the $^{15}$N chemical shift shown at the top of the panel (120.54 ppm). The spectrum of [U-$^{15}$N]$^2$H]-apo-IscU alone (red) is overlaid with the spectrum of [U-$^{15}$N]$^2$H]-apo-IscU-HscA (1:2; blue). The signals from Val-73 has disappeared in the spectrum of the complex, whereas that from Ile-93 is unperturbed. B, a double-plot of the $^1$H-$^{15}$N chemical shift of NMR signals corresponding to the D-state of IscU upon the addition of 2 eq of HscA plotted as a function of the residue number. Residues whose signals were observed initially but broadened and were no longer observed in the IscU-HscA complex are indicated (red $\times$). The L90PPVK106 motif known to interact with HscA (8, 9) is indicated in blue, and the $^{13}$TEWVKG106 motif that is proposed in this study as an additional recognition site is marked with an asterisk. C, secondary NMR chemical shifts ($\Delta\delta_{C\alpha} - \Delta\delta_{C\alpha}$) of IscU bound to HscA (red bars) are compared with those of apo-IscU in the structured (IscU(S); black bars) and disordered (IscU(D); blue bars) conformations. Although a few fewer NMR signals were visible in the complex, their secondary chemical shifts were similar to those of the D-state of IscU. The chemical shifts of the S- and D-states of IscU are from Biological Magnetic Resonance Data Bank accession numbers 17836 and 17837, respectively. These results and the chemical shift analysis of the assigned backbone signals of IscU indicate that the protein lacks secondary structure in the complex. D, perturbation pattern mapped onto the solution structure of the S-state of apo-IscU (Protein Data Bank 2L4X). IscU residues whose $^{1}$H-$^{15}$N NMR cross-peaks were observed in the IscU-HscA complex are colored green; residues whose cross-peaks broadened and were not observed by the interaction with HscA are colored red; and residues whose cross-peaks were not observed or assigned are colored blue.

**FIGURE 5.** Isolated SBD of HscA also binds and stabilizes the D-state of IscU. Left panel, two-dimensional $^{15}$N TROSY-HSQC spectrum of [U-$^{15}$N]$^2$H]-apo-IscU alone (red). Right panel, two-dimensional $^{15}$N TROSY-HSQC spectrum of [U-$^{15}$N]$^2$H]-apo-IscU and the presence of 2 eq of the unlabeled HscA SBD (blue) overlaid on the spectrum of apo-IscU (red). Peaks from the S-state of IscU are not seen in the spectrum of the complex. Most of the peaks in the complex resemble those of the D-state of IscU. However, a few new peaks at new positions (marked with arrows) appear in the spectrum of the complex; they likely correspond to IscU residues in the binding interface.

HscA SBD Preferentially Binds and Stabilizes the D-state of Apo-IscU—Studies of E. coli DnaK (31, 32) and the x-ray structure of the HscA SBD complexed with the nonapeptide E98LPPVKIHC106 (22) have shown that the isolated SBD binds its substrate. We carried out titration experiments similar to those described above, but with the HscA SBD in place of full-length HscA and with [U-$^{15}$N]$^2$H]-IscU, whose backbone $^{1}$H-$^{15}$N HSQC signals broadened much less than those of [U-$^{15}$N]IscU in the complex. The results demonstrated that the HscA SBD binds to and stabilizes a D-like state of IscU (Fig. 5). The addition of 2 eq of the HscA SBD led to the disappearance of the S-state peaks of apo-IscU. We conclude that the HscA SBD, like full-length HscA, binds to and stabilizes a D-like state of IscU. With the smaller complex containing fractionally deuterated IscU, we were able to resolve a few peaks (indicated by arrows in Fig. 5) that appear likely to correspond to residues of IscU directly involved in the binding interface.

Summary of Effects of Protein and Ligand Binding on the Conformational State of IscU—We found that the $^{1}$H-$^{15}$N cross-peaks from the backbone of Lys-128 in $^{15}$N TROSY-HSQC spectra of [U-$^{15}$N]apo-IscU provide a useful monitor for following the conformational state of IscU in protein-protein complexes (Fig. 6A). Although Lys-128 is located in the disordered C-terminal region of IscU, it nonetheless exhibits separate peaks corresponding to the S- and D-states of apo-IscU. In addition, because the residue is located in a highly disordered region, the intensities of its signals are minimally affected by the molecular size of the complex; and from its location and poor sequence conservation (16), it is unlikely that Lys-128 is involved in interactions with HscB or HscA. We measured the volumes of the peaks assigned to the S- and D-states under a variety of conditions and used these to calculate the following: percent structured (%S) = (100 × [S]/([S] + [D]) (Fig. 6B). %S $\approx$ 84 for 0.3 mM IscU alone. Upon the addition of equimolar HscB, %S increased to 93. Equimolar IscU + HscA had %S = 51, as did equimolar IscU + HscA-ADP. Equimolar IscU +
HscA(T212V) in the absence or presence of ADP yielded $\%S = 47$. $\%S$ increased to 51 for IscU + HscA(T212V) + ATP. Equimolar IscU + HscB + HscA had $\%S = 60$. This value remained about the same when wild-type HscA was substituted by HscA(T212V) or when ADP was added to equimolar IscU + HscB + HscA(T212V). However, $\%S$ increased to a value similar to that for IscU alone when ADP in the previous mixture was replaced by ATP. We used HscA(T212V), which lacks ATPase activity, as a surrogate for HscA in this experiment because the addition of IscU to HscA-ATP led to rapid ATP hydrolysis (data not shown).

These results confirmed our earlier finding that HscB binds preferentially to the S-state of IscU (16). The stabilizing effect of HscB is negated by HscA, which interacts preferentially with the D-state both alone and in its ADP complex. As noted above, the results establish that HscA-ATP fails to interact preferentially with either state of IscU.

**DISCUSSION**

An earlier model (30) postulated that cluster transfer occurs following the exchange of ADP with ATP, leading to release of holo-IscU from HscA. The results presented here show that cluster transfer instead is coupled to the hydrolysis of ATP bound to HscA with conversion of HscA from the T-state to the R-state, which binds IscU (Fig. 1). HscA-ADP binds to the D-state of IscU, and IscU in the complex is disordered and incapable of retaining the Fe-S cluster.

The results presented here, along with those published earlier (16), demonstrate that HscB binds preferentially to the S-state of IscU, i.e. the conformational state of IscU in its 2Fe-2S complex. The HscB-IscU-[2Fe-2S] complex then binds to HscA through interaction of the J-domain of HscB with the nucleic acid domain of HscA. However, because we found that the ATP-bound state of HscA failed to interact strongly with either the S- or D-state of IscU, we postulate that hydrolysis of ATP to ADP is necessary for initiating the interaction of HscA with IscU.

In the IscU-[2Fe-2S] complex, the side chains of His-105 and Cys-106 ligate different iron atoms, and the backbone conformation of the recognition peptide (E98LPPVKIHC106) is very different from its conformation when complexed to the HscA SBD (22). We found that, when bound to HscA, IscU residues flanking the recognition peptide exhibited NMR chemical shifts very similar to those of the D-state of IscU (Fig. 4). We conclude that IscU must undergo the S → D conversion to interact with HscA.

We speculate here that the attack of cysteinyl side chains from an acceptor protein on iron atoms of the IscU-bound Fe-S cluster is the event that triggers both ATP hydrolysis and the disordering of the recognition peptide that allows it to bind to HscA-ADP. In a possible scenario, which has yet to be tested, attack of two Cys residues from the acceptor protein on the two iron atoms of the [2Fe-2S] cluster displaces IscU ligands His-105 and Cys-106 in the recognition peptide. This reaction distorts the conformation of IscU and changes its interaction with HscB, which in turn transmits the change to the nucleotide-binding domain of HscA so as to turn on ATP hydrolysis. The ADP-bound form of HscA interacts with the recognition peptide and stabilizes the D-state of IscU, which releases the Fe-S
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cluster completely along with HscB. The net result is complete irreversible transfer of the cluster to the acceptor protein. Such a mechanism would have the benefit that ATP hydrolysis is triggered only when an acceptor protein is in place to receive the cluster, thus avoiding idle cycles of ATP hydrolysis and loss of the cluster without transfer.

It has been shown that Hsp70 proteins can disentangle non-native regions of a misfolded protein and assist folding to its native fold by means of an ATP-dependent cycle (33–35). Two mechanisms have been proposed to explain this effect: Hsp70 acts as an “unfoldase” by directly recognizing and unfolding a misfolded protein substrate and then releasing it (36), and/or Hsp70 acts as a “holdase” that prevents a polypeptide chain from being misfolded or aggregated by holding susceptible regions of the substrate (37). Our finding that HscA decreases the D → S rate and binds to and stabilizes the disordered state of IscU implies that it acts as a holdase, and our finding that HscA has no effect on the S → D rate and binds poorly to the structured state of IscU argues against its acting as an unfoldase. A major difference between HscA and the other Hsp70 proteins is that HscA is specifically targeted to the L39PPVK163 motif of IscU, whereas Hsp70 proteins usually have a wide range of substrate specificity. Our chemical shift perturbation study suggests that HscA may have a second recognition motif, V73TEWVKG79 (denoted by an asterisk in Fig. 4B). We identified signals from IscU in the complex (Fig. 5) that appear to correspond to residues directly involved in the binding interaction with HscA. Follow-up studies are under way to assign these signals.

IscU differs from the class of intrinsically disordered proteins that (partially) fold upon binding a protein or other ligand (38) in that the two states (D and S) are nearly equally populated in the absence of ligand. IscU belongs instead to the class of proteins termed metastable proteins (39), which, under physiological conditions, are capable of populating two different folds that fulfill different functions. Founding members of this class include lymphotactin (40, 41) and Mad2 (42). The two states of metastable proteins differ by <2 kcal/mol. Whereas the alternative states of lymphotactin and Mad2 are monomers and dimers, both states of IscU are monomeric. The co-evolution of stable alternative conformations in metamorphic proteins has yet to be explored in detail.

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