Metamorphic protein IscU alternates conformations in the course of its role as the scaffold protein for iron–sulfur cluster biosynthesis and delivery

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**A B S T R A C T**

IscU from *Escherichia coli*, the scaffold protein for iron-sulfur cluster biosynthesis and delivery, populates a complex energy landscape. IscU exists as two slowly interconverting species: one (S) is largely structured with all four peptidyl–prolyl bonds trans; the other (D) is partly disordered but contains an ordered domain that stabilizes two cis peptidyl–prolyl peptide bonds. At pH 8.0, the S-state is maximally populated at 25 °C, but its population decreases at higher or lower temperatures or at lower pH. The D-state binds preferentially to the cysteine desulfurase (IscS), which generates and transfers sulfur to IscU cysteine residues to form persulfides. The S-state is stabilized by Fe–S cluster binding and interacts preferentially with the DnaJ-type co-chaperone (HscB), which targets the holo-IscU:HscB complex to the DnaK-type chaperone (HscA) in its ATP-bound form. HscA is involved in delivery of Fe–S clusters to acceptor proteins by a mechanism dependent on ATP hydrolysis. Upon conversion of ATP to ADP, HscA binds the D-state of IscU ensuring release of the cluster and HscB. These findings have led to a more complete model for cluster biosynthesis and delivery.

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1. Introduction

Iron–sulfur (Fe–S) clusters are among the most ancient and ubiquitous protein prosthetic groups. They function in electron transport, enzymatic catalysis, chemical sensing reactions, or as structural units [1]. Cells have evolved elaborate mechanisms for producing iron–sulfur clusters so as to minimize exposure to their toxic constituents, iron and sulfur ions [2,3]. The biosynthesis of iron–sulfur clusters utilizes machinery that emerged very early in evolution and has remained highly conserved throughout biology. Humans and other eukaryotes utilize the ISC (iron–sulfur cluster) system as the essential Fe–S cluster assembly mechanism in mitochondria [4], and defects in this system have been linked to a large number of human diseases [5,6]. The prokaryotic ISC system has served as a useful model for understanding Fe–S cluster assembly and delivery [7,8]. The best-studied iron–sulfur cluster biosynthetic system is the ISC system of *Escherichia coli* (Fig. 1), which is highly homologous to the ISC system found in mammalian mitochondria. The IscU protein acts as a scaffold on which the Fe–S clusters are assembled and from which the clusters are delivered to various apoproteins. IscS is a PLP-dependent cysteine desulfurase that forms a heterotetrameric complex with IscU and transfers sulfane sulfur generated from the conversion of cysteine to alanine to the cluster ligand cysteines of IscU [9]. HscA and HscB, the DnaK-like chaperone and the DnaJ-like co-chaperone proteins, respectively, facilitate the Fe–S cluster delivery mechanism in an ATP-dependent manner [10]. Much of our understanding of these proteins has come from X-ray structures that have given a largely static view of the processes involved. We review here our recent studies by NMR spectroscopy and other biophysical approaches that show the importance of dynamic processes affecting these proteins, most notably IscU which we have found to populate two different conformations that interconvert on a second time scale: one conformation (S-state) is largely structured and the other (D-state) is partially dynamically disordered [11].

IscU is a highly conserved protein (Fig. 2). Conserved features include the cluster ligands and the site recognized by the chaperone (HscA). An early NMR study of *Thermotoga maritima* IscU suggested that it has features similar to a fluid molten globule and populates more than one state [12]. A subsequent publication revealed that *T. maritima* IscU exists “in a dynamic equilibrium
between two or more distinct conformations, possibly existing in a molten globule state,” with the data “not fully consistent with a rigid protein fold, a molten globule state, or a completely unstructured fold” [13]. Later NMR studies reported structural heterogeneity that could be resolved by adding Zn$^{2+}$ [14]. Another study characterized IscU as a ‘complex-orphan protein’ that is prone to unfolding if not stabilized by a co-factor or a protein partner [15]. We showed that apo-IscU exists in equilibrium between a structured (S) and disordered (D) state [11]. Our recent studies have revealed that the D-state consists of dynamically disordered and ordered domains, with the ordered domain stabilizing two high-energy cis peptidyl–prolyl bonds, which are trans in the S-state [16]. Thus, IscU can be classified as a metamorphic protein [17], a protein that can adopt two different folds. This raises the questions: why has IscU evolved to be metamorphic, and do the two different conformations play physiologically important roles?

We have found that single-site substitutions of conserved residues perturb the equilibrium between these two states, either favoring the S- or D-state [18]. We have further found that these substitutions interfere with the rate of cluster assembly or the stability of the assembled clusters [18]. The D-state of apo-IscU is the substrate for IscS [18]. Once the cluster is assembled, the IscU-[2Fe–2S] complex adopts the S-state conformation [19]. The co-chaperone (HscB), which targets IscU-[2Fe–2S] to the chaperone (HscA), binds preferentially to the S-state of IscU [11,20]. HscA and HscA(ADP) both bind preferentially the D-state, whereas HscA(ATP) does not bind tightly to either the D- or S-state [20]. Thus, our studies show that both states of IscU are physiologically important, and we have devised a working model for iron–sulfur cluster assembly and delivery that incorporates these findings (Fig. 3) [16]. We describe the evidence for features of this model in the following sections.

2. Characterization of the two interconverting states of IscU

The initial indication for the existence of two interconverting conformational states came from the detection of two $^1$H–$^{15}$N HSQC peaks from the side-chain $^1$H$^{15}$N of the single Trp residue (W76) of [U–$^{15}$N]-IscU (Fig. 4a). When Zn$^{2+}$ was added, the Trp peak at lower frequency disappeared as did the set of largely undispersed signals (Fig. 4b). The single-site variant IscU(D39A), which is known to stabilize the cluster in holo IscU, also yielded a set of signals (Fig. 4c) similar to those of the Zn$^{2+}$ complex (Fig. 4d). Thus we assigned the higher frequency Trp peak to the S-state and the other set of peaks to the D-state (Fig. 4e) [11]. Assignments of signals from the two states indicated that the largest chemical shift differences [11] between the S- and D-states.
were in residues corresponding to the two N-terminal β-strands and the C-terminal α-helix of the S-state of apo-IscU [21], the Zn\textsuperscript{2+} complex [14], or the [2Fe–2S] complex [19]. We found that the D-state lacks most of the secondary structure found in the S-state [18].

3. Structure of the S-state of IscU

We were able to determine the solution structure of the S-state of \textit{E. coli} IscU under conditions (pH 8.0, 25 °C, 20 mM Tris buffer) where the protein is 80% S and 20% D (pdb 2L4X) [21] (Fig. 5a and b). We also determined the solution structure of IscU(D39A), which is predominantly in the S-state, (pdb 2BKQ) [21] (Fig. 5c and d). The two structures are very similar except for the loops containing the residues that ligate the cluster, which are less disordered in IscU(D39A) and closer to the configuration seen in holo-IscU [19]. The fold of the S-state is similar to those of the zinc complexes of \textit{Haemophilus influenza} IscU (pdb 1R9P) [14] and \textit{Mus musculus} IscU (pdb 1WFZ) [22] and the structure of \textit{Aquifex aeolicus} [2Fe2S]-IscU(D39A) (pdb 2Z7E) [19].

4. Effects of pH and temperature on the D ↔ S equilibrium

The position of the D ↔ S equilibrium depends on the solution conditions. In solutions containing 50 mM Tris–HCl (pH 8.0), 0.5 mM EDTA, 5 mM DTT, 150 mM NaCl, 10% D\textsubscript{2}O, 50 μM DSS, and 50 μM NaN\textsubscript{3}, the highest level of the S-state, %S = ([S]/([S] + [D])) × 100, occurs at 25 °C (Fig. 6a) [23]. Raising or lowering the temperature leads to increased levels of the D-state. At 45 °C, the population of the S-state is near zero [16]. As the temperature is raised above 45 °C, the signals from the D-state diminish in intensity, and signals from unfolded protein increase. By 70 °C the protein is fully unfolded. These temperature-dependent conformational changes observed by NMR spectroscopy have been
lize the cluster-coordinating complex of IscU. This substitution was used to achieve the X-ray structure of A. aeolicus [2Fe–2S]-IscU [19]. This stabilization has been attributed to a decrease in solvent accessibility of the cluster [28,29]. As noted above, we explain the effect of the mutant in terms of stabilization of the S-state of the protein which binds the cluster. We have found other substitutions that stabilize either the S-state or the D-state (Table 1). As discussed below, these mutations provide useful ways of perturbing the D ↔ S equilibrium so that the distinct functional properties of the two states can be evaluated.

6. Evidence for difference in peptide bond isomerizations in the S- and D-states

Given the slow interconversion rate between the S- and D-states, which both have lifetimes on the order of one second [11], we suspected that the two states might differ by the cis–trans isomerization of a peptidyl–prolyl peptide bond. E. coli IscU has prolyl residues at positions 14, 35, 100, and 101. To investigate this, we prepared a sample of E. coli IscU labeled selectively with [U-13C,15N]-proline. To aid in the assignment of the NMR signals, we also incorporated [15N]-alanine. Given identities of the residues preceding proline (N13–P14, A34–P35, and L99–P100–P101), the labeled proline served to identify signals from P100 to P101 and the labeled alanine in addition to the labeled proline served to identify P35. Signals from P14 were assigned by exclusion [16]. We used two strategies to determine the configurations of the four peptidyl–prolyl peptide bonds in the S- and D-states: analysis of chemical shifts and NOEs. Previous studies of peptidyl–prolyl cis–trans isomerization have shown that prolyl residues with cis peptide bonds typically have 13C/C0 signals around 25 ppm and 13C/C0 signals near 35 ppm, with (δ13C/C0 – δ13C/C0) ≈ 10 ppm; conversely, prolyl residues with trans peptide bonds typically have 13C/C0 signals around 27 ppm and 13C/C0 signals near 32 ppm, with (δ13C/C0 – δ13C/C0) ≈ 5 ppm [30–34]. In the S-state, the chemical shift difference (δ13C/C0 – δ13C/C0) for all four prolyl residues was ~5 ppm, indicating that they are all trans (Fig. 7a). However, in the D-state, this chemical shift difference was ~5 ppm for P35 and P100, showing that they remain trans, but ~10 ppm for P14a and P14b (the two signals assigned to P14) and P101, indicating that they have become cis (Fig. 7b). In addition, we utilized the Promega program, which determines the statistical probability of a cis Xaa–Pro peptide bond from the sequence of the protein, the prolyl chemical shifts, and the backbone chemical shifts of neighboring residues [33]. The Promega analysis confirmed the assigned configurations for the prolyl peptide bonds at normalized likelihood values of greater than or equal to 0.99 [16]. The D-state itself exhibits local conformational heterogeneity in that two sets of signals were observed for cis P14 (P14a and P14b). The structural basis for this peak doubling currently is under investigation.

NOEs are also diagnostic for the configuration of a peptidyl–prolyl peptide bond. In a trans peptide bond the H2 of the preceding residue is close to the prolyl H2 and H3, whereas in a cis peptide bond the H2 of the preceding residue is close to the prolyl H1. NOE studies of IscU also confirmed that the N13–P14 and P100–P101 peptide bonds are both trans in the S-state and both cis in the D-state [16].

7. Evidence for preferential protein interactions with the S- and D-states

Given that apo-IscU exists in equilibrium between two states, S and D, it was of interest to determine whether one of these states interacts preferentially with the three different proteins it encounters during cluster assembly and delivery: IscS, HscB, and HscA. We
used three NMR approaches to investigate the interaction of the two states of IscU with the cysteine desulfsurfase (IscS) [18]: (i) we measured the effect of adding a sub-stoichiometric quantity of IscS on the S → D and D → S rates, (ii) we investigated the effect of added IscS on deuterium exchange at protected sites in the S-state of apo-IscU, and (iii) we determined the conformational state of added IscS on deuterium exchange at protected sites in the S-state, IscS ensures that the cysteine residues of IscU are unligated more slowly than wild-type IscU [18] (Fig. 9b). By selecting the D-state, IscS ensures that the cysteine residues of IscU are unligated by metals and available to react with the sulfur generated by the catalytic conversion of cysteine to alanine to form persulfides. With cluster formation, IscU converts to the S-state, which has a lower affinity for IscS and minimizes product inhibition.

Addition of the cochaperone (HscB) to IscU shifts the D → S equilibrium to the S-state [11,20]. This property ensures that HscB selectively recognizes holo-IscU, which is in the S-state, and is not tied up binding to the D-state. The J-protein domain of HscB binds to the ATPase domain of the chaperone protein (HscA), which is involved in cluster delivery to an acceptor protein by an ATPase-dependent process. We found that, whereas addition of HscA(ADP) to IscU failed to perturb the D → S equilibrium, HscA(ADP) shifted the equilibrium to the D-state [20] (Fig. 10). These observations suggest that binding of the D-state requires that HscA be capable of undergoing the conformational change induced by ATP hydrolysis (Fig. 10). Our experiments were carried out with IscU, rather than holo-IscU; however, it appears clear that the binding of HscA(ADP) to the D- rather than the S-state ensures that the cluster is released irreversibly from holo-IscU. The nonapeptide of IscU that is specific for binding to the substrate binding domain of HscA (ELPPVKIHC) contains two of the cluster ligands (H105 and C106), and the X-ray structure of the complex between this peptide and the substrate binding domain showed that the cluster ligands are not in a configuration where they could bind the cluster [37].

8. Working model for iron–sulfur cluster assembly and delivery

Our model for Fe–S cluster assembly (Fig. 3) has the D-state of IscU binding to the cysteine desulfsurfase (IscS) and sulfur transfer to cysteine residues of IscS to form persulfides prior to the addition of iron [18]. Upon the addition of iron, the atoms arrange to form a cluster which stabilizes the S-state of IscU [19]. The co-chaperone (HscB) then competes holo-IscU off of IscS (which does not bind the S-state of IscU strongly), and the His-Pro-Asp motif of its J-domain, which binds to the nucleotide binding domain of the chaperone (HscA) [38], is responsible for forming the HscA(ADP):HscB:holo-IscU complex. Our data indicate that the HscA(ADP) complex does not have an affinity for IscU [20]. Instead, we have speculated that the trigger that initiates ATP hydrolysis and cluster release under physiological conditions, most likely is the attack of one or more free sulfhydryl groups from the acceptor proteins on the iron atoms of the cluster bound to IscU [20]. Attack of one sulfhydryl group would displace one of the IscU ligands, and the weakest ligand (H105) could be the first to be displaced. H105 could serve as an acceptor for the proton released by the attacking SH. Protonation of H105, which favors conversion of the S-to-D-
state [23], could initiate a change in the conformation of the HscB:IscU complex that activates the ATPase through a change in the conformation of the bound J-domain. The change in conformation of HscA attending conversion of its bound ATP to ADP, generates the site that binds the D-state of IscU. The cluster would then be released from IscU to be bound fully by the ligands of the acceptor protein.

9. Outstanding questions and future prospects

Many questions remain concerning the detailed mechanism of iron–sulfur biosynthesis and delivery to acceptor proteins. Although the chemical exchange experiments described above suggest that the D \(\leftrightarrow\) S interchange is first order, recent unpublished results from our laboratory suggest that the two peptidyl–prolyl peptide bond interconversions may be sequential rather than concerted. This issue requires further examination. Electrons must be donated to the sulfur produced by the cysteine desulfurase in order to reduce the sulfur to produce the persulfide bound to IscU. We are currently investigating the potential role of the ferredoxin encoded by the isc operon (Fig. 1) as the electron donor. The physiological iron donor remains a mystery to be solved. Frataxin has been suggested as the iron donor[39], but the bacterial frataxin homologue, CyaY, has been found to inhibit cluster formation[40], whereas in the human system frataxin is required for optimal cluster assembly[41,42]. Although the hypothesis that cluster
delivery is triggered by attack of one or more cysteine side chains of the acceptor protein appears reasonable because it would ensure that transfer occurs only when an acceptor protein is at hand, it requires experimental verification. We also are studying yeast and human ISC proteins to determine whether they utilize similar mechanisms for iron–sulfur cluster assembly and delivery. Our preliminary investigations of the human homolog of IscU, ISCU, suggest that it is metamorphic like the E. coli protein (Kai C. and Markley J.L., unpublished).

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Fig. 9. Time course of iron–sulfur cluster assembly as monitored by absorbance at 456 nm at room temperature in an anaerobic chamber (Coy Laboratory) filled with 90% N2 and 10% H2. The cluster assembly reaction contained 50 μM apo-IscU, 1 μM IscS, 250 μM ferrous ammonium sulfate, 0.1 M Tris–HCl (pH 7.5), 5 mM DTT. The reaction was initiated by adding l-cysteine to achieve a concentration of 250 μM. (a) Cluster assembly with wild-type IscU (black). Reaction in the absence of IscS (orange) showing that iron–sulfur cluster was not assembled in the absence of the cysteine desulfurase. Cluster assembly in the presence 250 μM Zn(Cl)2 (scarlet) showing that added Zn2+ inhibits cluster assembly. (b) Comparison of the time course of cluster assembly on IscU variants. Wild-type (WT) IscU assembled clusters more efficiently than the other five variants studied. The more structured variants (E111A, S107A, and N90A) assembled clusters following an initial lag and at a slower rate than WT. Like WT, the less structured variants (K89A and N90D) did not exhibit an initial lag, but they assembled clusters at rates intermediate between WT and the more structured variants. The decay of intensity after its highest point arises from the instability of the cluster once formed. Figure adapted from [18].

Fig. 10. Conformational states of IscU under conditions mimicking various steps in the iron–sulfur cluster transfer mechanism. (Left) Backbone 1H–15N NMR peaks assigned to K128 in the D- and S-states of IscU. In each panel the peak from the D-state is on the left and that from the S-state is on the right. (Top panel) [15N]-IscU alone; (second panel) equimolar [15N]-IscU and HscB; (third panel) equimolar [15N]-IscU, HscB and HscA(T212 V) in the presence of excess ADP; (bottom panel) equimolar [15N]-IscU, HscB and HscA(T212 V) in the presence of excess ATP. (Right) To the right of each panel is a schematic figure representing the step being mimicked. Figure adapted from [20].


