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## Of the vulnerability of orphan complex proteins: The case study of the E. coli IscU and IscS proteins

Filippo Prischia, Chiara Pastorea, Marta Carronib, Clara Iannuzzia, Salvatore Adinolfia, Pierandrea Temussia, c, Annalisa Pastorea, ,

### Abstract

IscS and IscU, the two central protein components of the iron sulfur cluster assembly machinery, form a complex that is still relatively poorly characterized. In an attempt to standardize the purification of these proteins for structural studies we have developed a protocol to produce them individually in high concentration and purity. We show that IscS is a rather robust protein as long as it is produced in a PLP loaded form and that this co-factor is essential for fold stability and enzyme activity. In contrast to previous evidence, we also propose that, in contrast with previous evidence, IscU is a thermodynamically stable protein with a well defined fold but, when produced in isolation, is a 'complex-orphan protein' that is prone to unfolding if not stabilised by a co-factor or a protein partner. Our work will facilitate further structural and functional studies of these proteins and eventually lead to a better understanding of the whole machinery.

### Introduction

The concept of "molecular recognition" has gained a central role in structural biology especially since the completion of the human genome project has suggested the possibility that the secret of organism complexity could be in interactions [1]. In recent years, many structures of protein–protein and protein–ligand complexes have been solved by X-ray, nuclear magnetic resonance (NMR) and electron microscopy methods, pointing out that shape and electrostatic complementarity [2] ; [3] as well as hydrophobic contributions [4] play an important role in protein recognition. However, although the study of complex assemblies has become increasingly important, several difficulties remain intrinsic to the accomplishment of this task. A prerequisite for the study of molecular assemblies is the production of the proteins. This is often not easy to accomplish since some of the complex components are what we may call 'complex-orphan proteins'. With this term we may indicate proteins which, although not necessarily unstable from a thermodynamic point of view, tend to misfold or aggregate in the absence of their natural co-factors and/or protein partners or under conditions of environmental stress. This means that the study of these complexes requires the development of specific and well focused strategies.

Here, we discuss the problems encountered in a long-term systems biology project which aims at understanding the complex machinery required for iron sulfur cluster assembly and outline the ways we overcame some of these difficulties. In prokaryotes, the genes which encode the proteins involved in iron sulfur cluster assembly are conveniently grouped in distinct operons, known as *isc*, *suf* and *nif* [5]; [6]; [7] ; [8]. Each of these operons operates in different organisms and/or under different environmental conditions [5]. We are currently studying the gene products of the *isc* operon with the ultimate goal of dissecting and understanding the precise function of each of the different components. Among the up to nine *isc* proteins, a pivotal role is played by IscS, the desulfurase central to cluster production which converts cysteine into alanine and persulfide [9]. IscS forms a complex

with IscU, a scaffold protein which transiently hosts the cluster [10]. Although central to the iron sulfur cluster machinery still relatively little is known about the IscS/IscU complex.

In the present study, we focused on IscS and IscU as a paradigmatic example of complex-orphan proteins and discussed how to produce them in an active form. We also underline the dangers and the problems encountered when not succeeding in this task: it is clear from our experience that difficulties in protein production may lead to wrong or misleading interpretations.

## Materials and methods

### Protein production

The proteins were purified as previously reported [11]; [12] ; [13]. In short, IscU and IscS were subcloned by PCR from bacterial genomic DNA and individually expressed from a pET-30 vector (EMBL Heidelberg) as fusion proteins with His-tagged glutathione-S-transferase (GST) and a cleavage site for Tobacco Etch Virus (TEV) protease which leaves, after cleavage, two additional amino acids (Gly–Ala) at the protein N-terminus. IscS was also expressed from a pET-11 vector (EMBL Heidelberg). Unless otherwise specified, IscS was expressed in the presence of 100  $\mu$ M PLP purchased from Sigma and added without further purification to the growing media. IscU was expressed by growing the cells in LB enriched medium containing 8.3  $\mu$ M ZnSO<sub>4</sub> (Sigma) to stabilize its fold according to the protocol previously reported [14]. 15N- and 15N,13C-labelled samples of IscU for nuclear magnetic resonance (NMR) studies were produced by growing the bacteria in minimal medium using 15N ammonium sulfate and 13C-glucose as sole sources of nitrogen and carbon.

The proteins were purified by affinity chromatography using Ni–NTA gel and cleaved from the tag by TEV protease followed by Ni–NTA gel. All purification steps were carried out in the presence of 20 mM  $\beta$ -mercaptoethanol. The collected proteins were further purified by gel-filtration chromatography on a Superdex 75 26/60 column (GE Healthcare). Samples were eluted in a solution of 20 mM Tris-HCl buffer (pH 8.0), containing 150 mM NaCl and 20 mM  $\beta$ -mercaptoethanol.

Protein purity was checked by SDS–PAGE and by mass-spectrometry. Evaluation of the apparent molecular weight of IscS without PLP was achieved by size exclusion chromatography using Superdex 75 13/300 column (GE Healthcare).

Typically, we could obtain yields of 9 mg and 6.5 mg per liters of culture for unlabelled IscS and IscU, respectively.

### Isolation of the IscS/IscU complex

Analytical gel filtration experiments were performed using a prepacked HiLoad 10/30 Superdex 200 column (Pharmacia). The column was equilibrated with 20 mM Tris-HCl buffer (pH 8.0) in the presence of 150 mM NaCl and 20 mM  $\beta$ -mercaptoethanol. Samples were prepared by mixing 100  $\mu$ l IscS 0.4 mM and 150  $\mu$ l IscU 0.4 mM and bringing the volume to 500  $\mu$ l with sample buffer (20 mM Tris-HCl at pH 8.0, 150 mM NaCl and 20 mM  $\beta$ -mercaptoethanol). The final sample (80  $\mu$ M IscS and 120  $\mu$ M IscU) was loaded using a static

loop (500  $\mu$ l) and eluted with the same equilibrating buffer. The composition of the column fractions was analyzed by SDS–PAGE.

### Crystallization tests

To test whether IscS produced according to our protocol has a native fold we attempted crystallization under the conditions previously described [15]. IscS crystals were grown at 20 °C by vapor diffusion either in sitting or hanging drops, equilibrated against a solution containing 12% (w/v) PEG 10,000, 20% (w/v) PEG 2000, 0.1 M Tris-HCl pH 9 and 70 mM sodium citrate at pH 6.5. Crystallization trials for the IscS/IscU complexes were also set up using a variety of commercially available sparse-matrix screens. Crystallisation drops, containing either 1:1 or 1:2 volume ratios of protein to reservoir, were set up using the mosquito liquid handling robot (TTP LabTech's). Data were collected either at the X-ray crystallography facility of Imperial College London or at the microfocus beamline of the European Synchrotron Radiation Facility (ESRF, Grenoble, France). Processing of the images was performed using the Mosflm software.

### Assignment of the NMR spectrum of IscU

NMR spectra of Escherichia coli IscU were acquired on  $^{15}\text{N}$  or  $^{15}\text{N}/^{13}\text{C}$  uniformly labelled samples typically at 0.6 mM concentration in 90%/10%  $\text{H}_2\text{O}/\text{D}_2\text{O}$  and 20 mM Tris-HCl at pH 8.0, 150 mM NaCl and 10 mM DTT. All the spectra were recorded at 25 °C on Varian or Bruker spectrometers operating at 500, 600 and 800 MHz proton frequencies and equipped with 5 mm triple-resonance probes or cryo-probes. The WATERGATE sequence was used for water suppression [16]. The spectra were processed using NMRPipe [17] and analyzed using the Xeasy software [18]. The spectra were typically processed applying an exponential/Gauss window function and zero filled to double the size of the data.

The quality of the IscU NMR spectra is excellent. Complete assignment of most of the  $^1\text{H}$ ,  $^{15}\text{N}$ ,  $^{13}\text{C}$  backbone resonances was obtained using CBCA(CO)NH [19], CBCANH [19], HNCA [20], HN(CO)CA [21], and HNCO [22] recorded at 600 MHz. The assignment is deposited to the BMRB database (accession number 16,245).

### CD experiments

Secondary structure was checked by far-UV CD using a Jasco J-715 spectropolarimeter. The spectra were recorded at 25 °C using a cuvette with 0.1 cm pathlength. The protein concentration was 10  $\mu\text{M}$ . The buffer composition was 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 10 mM  $\beta$ -mercaptoethanol.

### NMR titrations

All spectra were recorded at 25 °C and 600 MHz using proteins in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 20 mM  $\beta$ -mercaptoethanol.  $^{15}\text{N}$  labelled IscU (0.4–0.6 mM) was titrated with aliquots of an IscS stock solution 0.55–0.6 mM to reach IscU:IscS molar ratios of 1:0.25, 1:0.5, 1:0.75, 1:1.

## Absorbance experiments

Cluster reconstitution was performed in an anaerobic chamber (Belle) under nitrogen atmosphere. Solutions of purified IscU (50  $\mu$ M) were incubated in sealed cuvettes typically using 3 mM DTT and 40  $\mu$ M Fe(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> for 30 minutes in 50 mM Tris-HCl buffer at pH 7.5 and 150 mM NaCl. Subsequently, 1  $\mu$ M IscS and 250  $\mu$ M Cys were added to start the reaction. Cluster formation was followed by absorbance spectroscopy using a Cary 50 Bio (Varian) spectrophotometer. Variations in the absorbance at 456 nm were measured as a function of time.

## Results

### IscS production

We produced IscS using two different vectors, a pET-11 plasmid with an N-terminal His-tag and a pET-30 plasmid with a GST-tag. In both cases, the FPLC1-chromatogram using a Superdex 75 26/60 column of IscS presented two distinct peaks (Fig. 1A). Only for the His-tagged protein, we could collect the two fractions separately and analyze them by SDS-PAGE, whereas due to peak overlap, we could not resolve IscS from the GST dimer since the two proteins do not differ enough in molecular weight.

IscS-PLP loaded purification. (A) FPLC-profiles of IscS loaded on a Superdex 75 ...

Fig. 1.

IscS-PLP loaded purification. (A) FPLC-profiles of IscS loaded on a Superdex 75 26/60 column after overnight TEV digestion recorded at 400 nm (gray) and 280 nm (black) wavelength. The peak at 145 ml elution is IscS-PLP loaded, since it absorbs at 400 nm. The peak at 165 ml corresponds to PLP free IscS. The protein concentration never exceeded 200  $\mu$ M. (B) Superposition of the absorption spectra of the gel filtration fractions eluted at 145 ml (gray) and 165 ml (black). (C) CD spectra of the PLP free (black) and loaded (gray) IscS samples.

Figure options

The two collected fractions both contained IscS, but had different absorption spectra (Fig. 1B). The species eluting at a volume compatible with the molecular weight of a dimer (90 kDa) presented an absorption peak around 390 nm, typical of the PLP group. A second peak had the typical absorption profile of a protein but no other absorption suggesting lack of PLP. This peak eluted at an apparent molecular weight of 65 kDa, that is very distinct from that expected for either the dimer (90 kDa) or the monomer (45 kDa). CD spectra revealed that the fraction lacking PLP is completely unfolded whereas the 90 kDa species, which is PLP loaded, is folded (Fig. 1C). This species also proved to be stable as a function of time and less prone to aggregation.

Therefore, we concluded that IscS needs PLP to be in a correctly folded dimeric state, its fold depending on the presence of the co-factor.

### Estimate of PLP occupancy in IscS

To increase purity and homogeneity of our IscS samples and standardize their production, we screened different purification protocols to increase PLP occupancy. Few protocols have been published on the evaluation of the occupancy in enzymes of PLP or similar cofactors.

Occupancy is commonly estimated by extracting the cofactor from the macromolecules using strong acids [23]. Alternatively, Vickery and coworkers estimated occupancy of ca. 90% from sample homogeneity [9]. We used a different approach.

We calculated the occupancy from the molar ratio between the protein (at 280 nm) and the bound PLP (at 390 nm) using the formula:

$$\text{Occupancy} = (A_{280} / \epsilon_{\text{IscS}}) / (A_{390} / \epsilon_{\text{PLP}})$$

Turn MathJax on

where  $A$  is the absorbance at the corresponding wavelength and  $\epsilon$  is the extinction coefficient. This value for PLP was obtained as the slope of free PLP concentration as a function of absorbance at 410 nm (Fig. 2A). We could safely assume that the band at 390 nm observed in PLP-loaded IscS solely corresponds to bound PLP using the following assumptions. The absorbance spectrum of unbound PLP has absorption peaks at 410 nm and 295 nm depending on the pH, which correspond respectively to the active unprotonated and the inactive protonated forms [23]. The absorbance maximum of PLP-loaded IscS around 390 nm must correspond to the 410 nm band upshifted because it is in a buried environment. The spectrum of IscS in a PLP free misfolded form does not absorb at this wavelength. Under these conditions, no free PLP is present in solution as we could check by comparing the 1D NMR spectra of IscS and of free PLP (Fig. 2B): the spectrum of PLP contains a characteristic sharp peak at 2.7 ppm which disappears completely in the protein spectrum, as expected when the co-factor is bound to the high molecular weight IscS dimer.

Estimate of the PLP occupancy in IscS. (A) Calibration curve to estimate the PLP ...  
Fig. 2.

Estimate of the PLP occupancy in IscS. (A) Calibration curve to estimate the PLP extinction coefficient obtained by recording the PLP concentration versus the absorbance at 410 nm. (B) Comparison of the NMR spectra of PLP and of a PLP-loaded IscS sample. The sharp resonance at 2.7 ppm corresponds to PLP and disappears completely in the spectrum of the protein indicating that all the PLP is bound to IscS. The resonance at 3.8 ppm arises from the buffer (Tris-HCl). (C) IscS crystals as obtained in 12% (w/v) PEG 10000, 20% (w/v) PEG 2000, 0.1 M Tris-HCl at pH 9 and 70 mM sodium citrate at pH 6.5 as previously described [15].

Figure options

We applied this protocol to estimate the PLP ratio of different IscS preparations purified following the same procedure. We found a 1:0.4 IscS:PLP molar ratio when cells expressing the GST-tagged protein were grown in LB medium. This is mainly due to the inefficiency of the purification protocol, which does not allow separation of the unfolded PLP-free form from the folded PLP loaded protein. The occupancy was 1:0.9 for the GST-tagged protein produced from cells grown in a PLP enriched LB medium. This indicates that the presence of PLP in the medium increases the occupancy but the higher ratio of IscS:PLP was almost counter-balanced by a toxic effect of PLP onto the cells. This significantly reduced the production yields. Only for the His-tagged protein obtained from cells grown in LB medium did we obtain a 1:1 occupancy. This result indicates that the ability of efficiently discriminating between the folded dimer and the unfolded monomer allowed us to obtain a pure 100% PLP loaded sample.

Crystals of IscS obtained in this way could be easily reproduced under the same conditions described by Vickery et al. [15] (PDB entry code: 1P3 W) (Fig. 2C). This indicates that the purified protein is properly folded and suitable for further structural studies of IscS in complex with its partners IscU, CyaY and YfhJ.

### IscU production

Production of IscU proved to be more problematic. During the nearly eight years of studies of this protein, we observed a large variability of the NMR spectra of IscU depending on the preparation used. In many of our samples, we observed more peaks than expected. For example, we could deduce the presence of two distinct tryptophan residues whereas we would expect only one from the protein sequence (Fig. 3A). The resonance of one of the two indole amide groups is at the position typical for a random coil structure. This result indicated the presence in solution of at least two species, one well folded, the second one being unfolded.

Comparison of IscU produced in different ways. (A) NMR HSQC spectra of IscU ...  
Fig. 3.

Comparison of IscU produced in different ways. (A) NMR HSQC spectra of IscU grown without addition of zinc in the medium. The two resonances corresponding to the two indole Trp are boxed. (B) The same as in (A) but grown in the presence of zinc. (C) Final point of a titration of IscU with an equimolar ratio of IscS. The spectrum of IscU disappears indicating formation of the complex. The spectra were all recorded at 25° C and 600 MHz.

#### Figure options

The amount of the unfolded species decreased when we started adding zinc in the growth media, supporting the suggestion that this cation stabilizes the fold by compensating for the absence of the cluster and/or of the binding partner IscS [24]. The HSQC spectrum of samples prepared in this way is that of a well folded monomeric protein with well spread sharp resonances (Fig. 3B).

When sample production became fully reproducible, we could fully assign most of the backbone resonances, that is out of a total of 124 non-proline residues (there are four prolines) 97 residues were assigned. The resonances of the residues which remained unassigned are either absent in the spectrum or do not form connectivities with the neighbouring groups thus making the assignment difficult. Among the non-assigned resonances are the first 16 N-terminal residues which is unfolded in other PDB deposited NMR structures and those of residues 21–23, 35, 36, 49, 64, 65, 100, 101, 104 and 105 which are in loop regions.

These observations strongly suggest that, even when correctly folded, the N-terminus of the protein is more flexible and unstructured.

### Studying the IscU/IscS interaction by NMR

To prove that the two proteins interact with each other as previously described for other orthologues [9]; [25]; [26], we followed the effect of titrating unlabelled PLP-loaded IscS into <sup>15</sup>N labelled IscU by hetero-nuclear NMR techniques. The IscU spectrum is affected by



titration with IscS already at a 1:0.7 IscU:IscS molar ratios confirming the presence of a specific interaction between the two proteins. At a 1:1 molar ratio we observe disappearance of nearly the whole spectrum (Fig. 3C). No indication of precipitation was found. Since the resonances disappear without concomitant chemical shift perturbation we must conclude that the effect is due to complex formation and that the process is under an intermediate-slow exchange regime in the NMR time range.

#### Isolation of a stable IscU/IscS complex

A 50% molar excess of IscU was added to a 0.4 mM solution of IscS and the mixture was analyzed by analytical gel filtration. The resulting profile differs from that of the single components, suggesting formation of the complex (Fig. 4A). This species could be independently purified and analyzed by SDS-PAGE (Fig. 4B). Fractions collected from the first high molecular mass peak of the gel filtration profile contain the complex. No fraction containing only IscS was found. Fractions from the second peak correspond to a lower molecular mass and contain only IscU. This result was expected since IscU was present in excess in the initial sample.

Isolation of IscS–IscU complex. (A) FPLC-profile of a mixture of the two ...

Fig. 4.

Isolation of IscS–IscU complex. (A) FPLC-profile of a mixture of the two components. The peak eluting at 7.8 ml corresponds to the IscS/IscU complex, the peak at 11.5 ml is isolated IscU (added in excess). (B) SDS-PAGE gel of all the fractions collected between 6 and 12 ml. (C) Comparison of the enzymatic rates of iron sulfur cluster formation on IscU as followed by absorption spectroscopy at 456 nm. The black curve was obtained using IscS with a high PLP occupancy. The gray curve was recorded using IscS purified from a GST-fusion construct (low PLP occupancy).

Figure options

Crystallisation trials using the IscS/IscU complex were setup but were unsuccessful. Two likely reasons for it are the relatively low affinity of the complex [9] and the flexibility of the N-terminal tail of IscU.

#### Efficient production of IscS and IscU appreciably influences the enzyme kinetics

We used IscS and IscU to study the kinetics of Fe–S cluster formation on IscU and establish a detailed mechanism [27]. Therefore we tested how different protocols of purification could influence our results (Fig. 4C). When IscS was prepared with the protocol which ensures high PLP occupancy we obtained appreciably faster kinetics, both confirming the active state of the protein and giving us an estimate of the variability we may expect in this assay when in suboptimal conditions.

#### Discussion

The IscU/IscS complex is central to iron sulfur cluster formation: IscS is the enzyme which provides the sulfur essential for the cluster, whereas IscU is widely accepted to be the transient acceptor scaffold [26]. The two proteins are known to form a complex [9] ; [26] thus suggesting a specific relationship. Complex formation is undoubtedly advantageous to allow efficient transfer of the persulfide from the enzyme to the scaffold. Much however

needs to be understood about the mechanism of cluster assembly and this is why it is essential to be able to produce isc proteins in a reproducible active form.

We have shown in this study that IscS is a relatively robust protein as long as it retains its PLP co-factor which we have shown to be essential for protein fold. We have provided guidelines both on how to produce the protein in a substantially populated PLP loaded form and how to estimate the co-factor content. The protein produced in this way is correctly folded, enzymatically active and able to crystallise reproducibly.

Obtaining a conformationally active form of IscU is more difficult, since IscU is a relatively small protein with a limited hydrophobic core and is prone to misfolding and unfolding. While thermodynamically not unstable as shown by previous studies [12], we have observed that its production in isolation can be problematic. This is in full agreement with other authors' observations [24] although the interpretation of some of the data may be slightly different. IscU is in our opinion incorrectly considered an intrinsically unstable protein or a molten globule state. We have shown in this and in a previous study that most of the apparent instability of IscU can be explained by the absence of a suitable partner. In vivo, IscU is embedded into an anaerobic environment which would prevent, for instance, oxidation of the three well conserved but exposed and reactive cysteines [10]. It will also be in an IscS bound form state or, when released from this interaction, will likely be loaded with an iron sulfur cluster.

The difficulty of handling orphan complex proteins may have led structural biologists to overinterpreting the problematic data of some of these proteins. For instance, *Thermotoga maritima* IscU has been depicted as displaying unique structural and motional characteristics with respect to other members of the same class of proteins [28]. These authors suggested that IscU from *Thermotoga maritima*, unlike other proteins able to host iron sulfur clusters, adopts a mobile, molten globule-like state and attributed to this state specific physiological relevance, implying that the extra flexibility might be necessary to bind the iron sulfur cluster. As stated above, this view is at variance with our own experience with *E. coli* IscU. It is true that, when isolated from its native complex, IscU is particularly vulnerable and may require the help of cofactors, such as the zinc ion, to remain stable for a prolonged time. However, it appears to be a well folded stable protein when properly expressed and isolated [12] even though we cannot exclude that an unfolded state might have a functional significance in nature.

We suggest that the apparent instability of IscU can be explained by classifying it as a complex-orphan protein that is a protein difficult to produce in the absence of its cellular partners. We previously introduced this concept for FMRP, a protein which is hard to produce and very prone to aggregation and/or degradation [29]. As IscU, also FMRP is known to take part to a complex network of interactions with other cellular partners. We strongly believe that the family of the complex-orphan proteins, well distinct from other classifications, is of high interest for understanding molecular assemblies.

In conclusion, we have discussed here the problems in producing two biologically essential proteins when separated from their native complex status. It is interesting to notice that difficulties in producing isolated proteins of the isc operon have been observed also for

other components. The alternative scaffold isc protein IscA, for instance, can be obtained in a correctly folded iron sulfur cluster loaded state only when co-expressed with other members of the operon [30]. This is certainly an important and promising direction which we are going to pursue next.

#### Note added in proof

While this manuscript was under review, a paper was published describing the crystal structure of the IscS/IscU complex from *E. coli* (Shi, R., Proteau, A., Villaroya, M., Moukadiri, I., Zhang, L., Trempe, J.F., Matte, A., Armengod, M.E., Cygler, M. (2010) *PLoS Biol.* 8, e1000354).

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