

Original Study

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Alberto Barbiroli, Francesco Bonomi, Stefania Iametti*, Mauro Marengo

Stabilization of the “open” conformer of apoIscU on the surface of polystyrene nanobeads accelerates assembly of a 2Fe2S structure

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Abstract: The scaffold protein IScU is involved in the assembly/transfer of FeS clusters. IScU exists in both open and closed conformation. The clusterless open conformation of IScU adheres to the hydrophobic surface of polystyrene nanobeads, as observed for other proteins. Increased accessibility of the ligand cysteines in bound IScU facilitates assembly of a 2Fe2S cluster, and the cluster-bearing structured form of IScU does not interact with the nanobeads, thus ensuring turnover. The dependence of accelerated cluster assembly on the nanobeads concentration pointed to steric and crowding effects as for promoting cluster formation, and confirms the requirement for structural flexibility of IScU.

1 Introduction

The well characterized *isc* (iron-sulfur cluster) operon in *E. coli* encodes for an array of mutually interacting proteins involved in the biogenesis of iron-sulfur structures, that represent one of the most common and relevant bio-inorganic cofactors, and appear extremely conserved all through evolution [1]. Indeed, similar systems are active in mitochondria, and all include - among others - the scaffold protein IScU (or its orthologs). In all IScU-type proteins a metastable FeS cluster is assembled from a yet unknown source of iron, and from sulfide derived from a cysteine thiol through the action of the pyridoxal phosphate-dependent sulfurtransferase IScS [2-5]. The 2Fe2S cluster formed on IScU may then be donated to acceptor apoproteins, or converted through suitable redox chemistry into FeS structures of higher nuclearity [6-8]. A specialized *isc*-

encoded system consisting of chaperones/cochaperones HscA/HscB stimulates in ATP-dependent manner the transfer of a 2Fe2S cluster from the cluster-loaded holoform of IScU (holoIscU) to suitable acceptors of 2Fe2S clusters [9,10]. However, the clusterless form of IScU (apoIscU) was also reported to catalyze the “in vitro” insertion of a 2Fe2S cluster on an acceptor apoprotein in the presence of iron salts and inorganic sulfide as the sources of the cluster atoms, without the formation of spectroscopically detectable intermediate clusters on IScU itself [11].

All these activities have been shown to be associated to a remarkable structural flexibility of IScU, that may be modulated by interaction with other components of the system. For instance, interaction with HscB stabilizes a rigid and folded conformation of apoIscU [12], resulting in a more difficult cluster assembly on IScU and an impaired transfer of the cluster to acceptor apoproteins, unless HscA and ATP are present [Barbiroli, Iametti and Bonomi, submitted]. The same types of impairment was observed for a structurally rigid D39A mutant of IScU. Other studies have reported that IScU retains a stable conformation when interacting with other protein partners relevant to the biosynthetic process [13], whereas distortion of the cluster ligation on IScU has been demonstrated to be essential to chaperone/ATP dependent acceleration of cluster transfer. On these bases, it has been suggested that forms of IScU with different structural rigidity play specific roles in their functions during FeS biosynthesis [12].

In separate studies, some of us have reported that proteins may unfold rapidly and spontaneously upon contacting the hydrophobic surface of polystyrene nanobeads [14], thus exposing reactive groups that are otherwise buried in the native protein structure. In the case of the apo-forms of metal-containing proteins, exposure of reactive groups may facilitate uptake of the metal, and metal uptake then in turn triggers protein refolding. Upon refolding around the newly inserted metal, the protein loses its ability to stick to the nanobeads, and is released in solution as the folded, metal-loaded holoform

*Corresponding author **Stefania Iametti**, DeFENS, University of Milan, Via Celoria 2, 20133, Milan, Italy,
E-mail: stefania.iametti@unimi.it

Alberto Barbiroli, Francesco Bonomi, Mauro Marengo, Section of Chemical and Biomolecular Sciences, DeFENS, University of Milan

[15]. Thus, polystyrene nanobeads behave as “biomimetic chaperones” as for catalyzing metalloprotein folding.

In this work, we have examined the possibility that stabilization of an unfolded form of apo-IscU on the surface of polystyrene nanobeads could facilitate the formation of a cluster-loaded form of IscU. For the sake of simplicity, this preliminary study was limited to chemical reconstitution experiments, using lithium sulfide and ferric salts as the source of cluster atoms. However, in principle, this approach could be extended to verify whether the interaction between IscU and the surface of polystyrene nanobeads may affect the equilibrium among IscU conformers [12,13], and therefore other functions of IscU, including the ability to interact with other partners in the cluster assembly process (such as the cysteine desulfurase IscS or the isc-encoded ferredoxin [12,16]), and/or with acceptor proteins. This latter aspect is possibly relevant to specificity issues, and could be of relevance to human pathologies [17].

2 Methods

Recombinant *E. coli* IscU was prepared and isolated as the apo-form according to published procedures [18]. Sample manipulation and reagent preparation were carried out anaerobically under Ar in septum-capped vials or cuvettes, and stainless steel needles and cannulae were used for sample transfer. Chemical reconstitution reactions were started by adding 0.1 mM Li₂S to a solution containing 40–45 μM apoIscU and 0.1 mM ferric ammonium citrate in 0.1 M Tris-HCl, pH 8.0, and 5 mM dithiothreitol. Cluster assembly was measured at 23°C by monitoring the increase in ellipticity at 435 nm [9–11] in 1-cm pathlength cuvettes, using a Jasco J-810 spectropolarimeter. When indicated, the given concentrations of polystyrene nanobeads (Kisker Biotech, nominal diameter 46 nm) were added to the reaction mixture prior to the reagents used to start cluster assembly. No changes in ellipticity were observed when IscU was omitted from these mixtures, thus ruling out the possibility of FeS clusters being assembled from iron salts and inorganic sulfide on the nanobeads surface.

3 Results

Addition of stoichiometric amounts of lithium sulfide to a buffered solution containing apo-IscU, dithiothreitol and stoichiometric iron salts results in the progressive appearance of the signature CD features of [2Fe2S]IscU, that are centered at ≈ 435 nm in the visible region of the CD spectrum [9–11]. As shown in Fig. 1, the time course of

cluster formation on IscU – as detected by CD spectroscopy – was markedly accelerated by the presence of polystyrene nanobeads. It has to be noted that – given an estimated molecular mass of 30×10^6 Da for the nanoparticles used here – the maximum concentration of nanobeads (0.4 g L^{-1}) does not exceed 12 nM. This keeps the maximum molar ratio between nanobeads and IscU lower than 1/4000. The same 46 nm nanobeads – having an estimated individual surface of $\approx 6 \times 10^{-9} \text{ mm}^2$ – were found to be saturated with 100–200 copies of bovine milk beta-lactoglobulin, a protein having size and charge comparable to those of apoIscU, but a much stiffer structure [14].

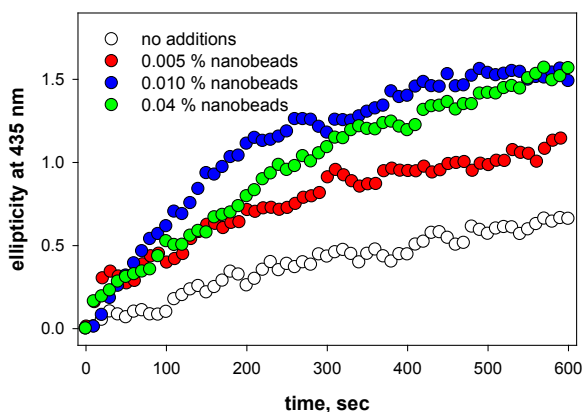


Fig. 1. Time course of the appearance of the CD signal of IscU[2Fe2S] in mixtures of apoIscU, dithiothreitol, ferric ammonium citrate, and Li₂S, containing the given NP concentrations.

Therefore, the amount of apoIscU present in non-bound form in solution is expected to be roughly three orders of magnitude higher than that of the protein absorbed on the polystyrene nanobeads. This suggests that the acceleration observed in Fig. 1 involves a turnover of the (apo)protein on the surface of polystyrene nanobeads. This turnover may be explained by assuming that the holoform of IscU is unable to maintain its interaction with the surface of polystyrene nanobeads, as already observed in refolding studies on other metalloproteins [15]. These same studies indicated that iron salts (either ferric or ferrous) do not bind to the nanobeads. Thus, the observed acceleration does not depend on an increased reagent concentration on the nanobeads surface. Also, there was no evidence in these experiment for the appearance of CD signals prior to the addition of sulfide, ruling out the possibility that the iron was bound to IscU (in the absence of sulfide) to give a structured rubredoxin-like geometry [18].

Indeed, as shown in Fig. 2, the spectral features of the proteins obtained in the presence/absence of polystyrene nanobeads were similar, although they had different

intensity when measured at early reaction times. However, the CD spectra of the various mixtures were essentially overlapping at equilibrium (180 min), indicating that the presence of the nanoparticles only had an impact on the rate of cluster assembly, but not on the reaction outcome. Previous studies on the interaction between IscU and HscB indicated that formation of a 1:1 complex between the two proteins shifted the solution equilibrium towards a rigid and folded conformation of apoIscU, resulting in much slower rates of formation of a CD-active cluster on IscU [Barbiroli, Bonomi, Iametti, submitted], but again with no reported effects on the complete formation of (2Fe2S)IscU at equilibrium. At contrast, the evidence presented here suggests that interaction with the surface of polystyrene nanobeads stabilizes an open and flexible conformation of IscU, on which assembly of a CD-active 2Fe2S cluster is facilitated.

Fig. 3 makes it evident that the “catalytic” effect of polystyrene nanobeads with respect to the initial rate of the cluster assembly reaction, that did not increase linearly with the concentration of the nanobeads, but reached apparent saturation at molar ratios nanobeads:IscU around 1:7500 (that is, at 0.2 g L^{-1} nanobeads and 0.6 g L^{-1} monomer IscU). A similar “saturation” behavior was reported for nanobead-facilitated refolding of other metalloproteins upon metal ligation, and was ascribed to molecular crowding effects [13]. In other words, increasing the nanobeads concentration above a given threshold increases the hydrophobic surface available for binding of the protein, and results in a modified orientation of the bound protein with respect to the one possible at

higher protein/nanobeads ratio. We hypothesize that the orientation favored at low protein/nanobeads ratios is less conducive to cluster assembly and insertion.

4 Discussion

The highly schematic representation in Fig. 4 provides a possible hypothetical rationale for the observed nanobeads-dependent acceleration of the cluster assembly process (Fig. 4A) and for the role played by molecular crowding in explaining the non-linear dependence of acceleration on the nanobeads concentration (Fig. 4B). Indeed, up to a specific concentration of nanobeads, binding of apoIscU to the nanobeads surface occurs with a “productive” orientation, which is favored by the scarcity of available binding surface with respect to the abundance of protein. As the concentration of nanobeads is increased, also the concentration of “productively oriented” bound protein increases, resulting in acceleration of the cluster assembly process. However, when the concentration of nanobeads exceeds a certain threshold, it will be possible for apoproteins to adhere to the nanobeads surface with a different orientation (and affinity), thus affecting both the possible geometric relationships among neighboring proteins on the nanobead surface and/or the rate of displacement/replacement of (2Fe2S)IscU by the incoming apoIscU.

In conclusion, we hypothesize that binding of the “open” conformer of apoIscU to the nanobeads surface: 1) shifts the conformational equilibrium of IscU towards

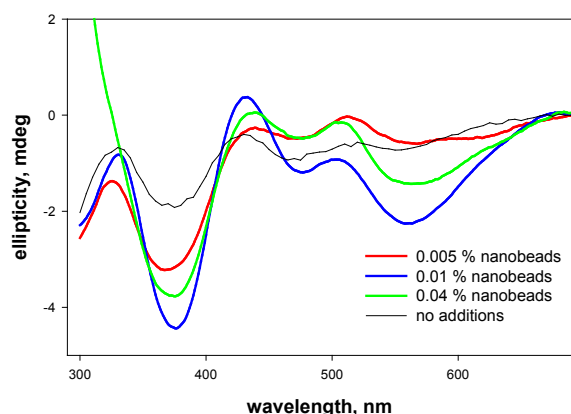


Fig. 2. CD spectra of mixtures of apoIscU, dithiothreitol, ferric ammonium sulfate, Li_2S , and the given nanobeads concentrations, taken 10 min after the reaction onset (all spectra were identical at 180 min).

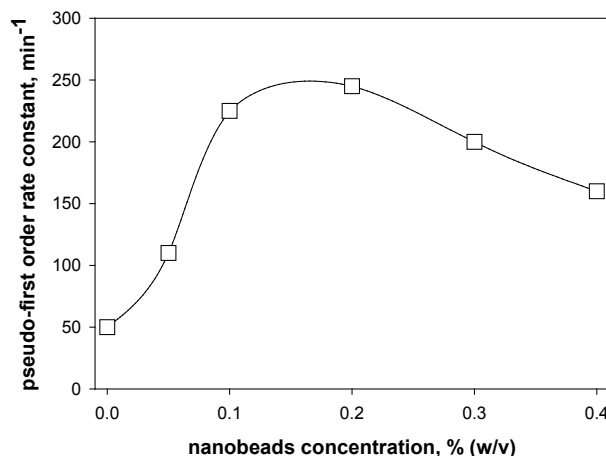


Fig. 3. Dependence of the pseudo-first order rate of appearance of the CD signal of IscU[2Fe2S] on the nanobeads concentration.

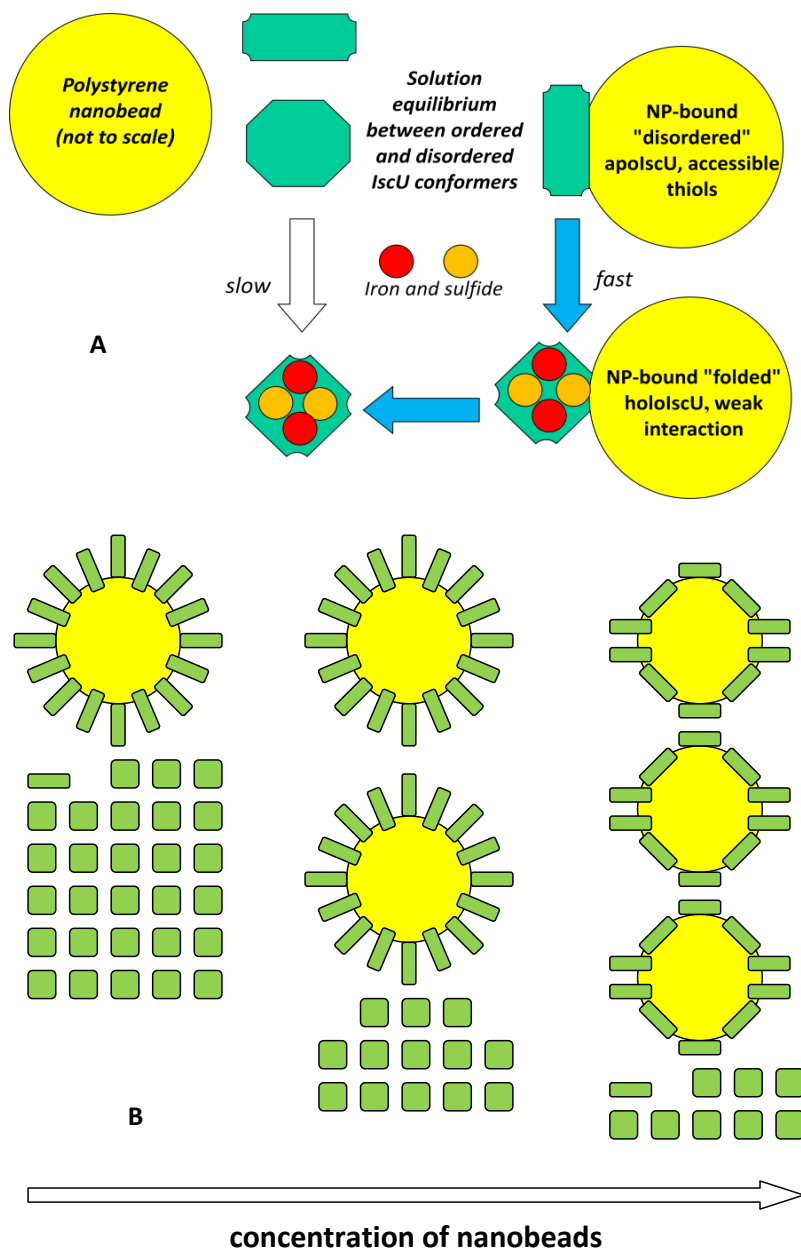


Fig. 4. Schematic representations of: A) the effects of nanobeads on the equilibrium and rates of cluster assembly on IscU; B) the possible role of “crowding” effects in generating properly unfolded forms of IscU.

the “open” conformation, thus facilitating uptake of the cluster atoms; 2) brings close to each other individual IscU molecules, thus facilitating formation of a cluster-bridged dimer (see below, [18]; 3) that is sensitive to the protein/nanobeads ratio, as lowering this ratio may favor an interaction geometry that is not conducive to facilitated cluster assembly.

In the peculiar case of IscU, geometry of protein-protein interactions is of particular relevance, as the formation of a CD-active [2Fe₂S]₂IscU has been suggested to

relate to formation of a cluster-bridged IscU dimer [9,10]. However, only one of the two monomers in [2Fe₂S]₂IscU is the substrate for chaperone-controlled cluster release to acceptor apoproteins [10]. Formation of a cluster-bridged IscU homodimer is likely to be accelerated when proper interactions among involved species (that is, between IscU monomers and among them and the cluster ions) do not depend on random encounters in solution, but occur with a more appropriate and productive geometry/orientation on the surface of polystyrene nanobeads. This is the

species that is detectable by CD spectroscopy, and that - once formed - is no longer able to adhere to the surface of nanobeads. Thus, its rate of formation may dictate the rate of turnover of the proteins on the surface of nanobeads.

The preliminary evidence provided here indicates that presence of an unstructured IscU could be a necessary pre-requirement for cluster assembly, and that [2Fe2S]IscU is structured and exposes no hydrophobic surface regions. This is circumstantially made evident by the observation that [2Fe2S]IscU is not capable of interacting with the hydrophobic surface of the polystyrene nanobeads.

From the standpoint of understanding the physiological aspects of the whole process, the ability of polystyrene nanobeads to trap the open IscU conformer could be conducive to address some of the issues related to transfer of the cluster atoms to the protein under physiological conditions, where interaction with other protein components of the assembly system comes into play, also because of issues related to the accompanying redox chemistry [12,13,16]. The observations reported here may also help in clarifying some of the much obscure mechanistic aspects of the reported “catalytic” activity of IscU [11].

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