ARTICLE IN PRESS

BBAMCR-17394; No. of pages: 9; 4C: 3, 4, 5, 6, 7

Biochimica et Biophysica Acta xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Anatomy of an iron-sulfur cluster scaffold protein: Understanding the determinants of [2Fe–2S] cluster stability on IscU☆

Miquel Adrover [a], Barry D. Howes [b], Clara Iannuzzi [c], Giulietta Smulevich [b], Annalisa Pastore [d,]*

[a] IUNICS, Departament de Química, Universitat de les Illes Balears, Crta. Valldemossa, km 7.5, E-07122 Palma de Mallorca (Spain)
[b] Dipartimento di Chimica "Ugo Schiff", Università di Firenze, Via della Lastruccia 3-13, I-50019 Sesto Fiorentino (FI), Italy
[c] Department of Biochemistry, Biophysics and General Pathology, Seconda Universita' di Napoli, Via De Crecchio 7, 80138 Naples, (Italy)
[d] Department of Clinical Neurosciences, King's College London, Denmark Hill Campus, London SE5, (UK)

A R T I C L E I N F O

Article history:
Received 14 July 2014
Received in revised form 22 October 2014
Accepted 28 October 2014
Available online xxxx

Keywords:
Iron–sulfur cluster
isc operon
QM/MM methods
Scaffold protein

A B S T R A C T

Protein-bound iron sulfur clusters are prosthetic groups involved in several metabolic pathways. Understanding how they interact with the host protein and which factors influence their stability is therefore an important goal in biology. Here, we have addressed this question by studying the determinants of the 2Fe–2S cluster stability in the IscU/Isu protein scaffold. Through a detailed computational study based on a mixed quantum and classical mechanics approach, we predict that the simultaneous presence of two conserved residues, D39 and H105, has a conflicting role in cluster coordination which results in destabilizing cluster-loaded IscU/Isu according to a 'tug-of-war' mechanism. The effect is absent in the D39A mutant already known to host the cluster more stably. Our theoretical conclusions are directly supported by experimental data, also obtained from the H105A mutant, which has properties intermediate between the wild-type and the D39A mutant. This article is part of a Special Issue entitled: Fe/S proteins: Analysis, structure, function, biogenesis and diseases.

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1. Introduction

Prokaryotic IscU and its eukaryotic Isu ortholog form one of the most conserved and widely spread protein families found in nature [1]. They are essential proteins involved in the assembly of iron–sulfur (Fe–S) clusters, a prosthetic group implicated in a wide variety of biological functions from electron transport to structural roles, to catalysis [2,3]. It is generally accepted that IscU/Isu acts in combination with IscS/Nfs1 [4,5], a desulfurase that converts cysteine into alanine by formation of a highly reactive persulfide [6]. Two IscU/Isu monomers independently bind the IscS/Nfs1 dimer and act as the 'preferential' partners to which the enzyme transfers persulfide for cluster formation. IscU/Isu is thought to bind both 2Fe–2S and 4Fe–4S clusters [7,8]. However, 2Fe–2S clusters assemble directly on the IscU/Isu monomer bound to IscS/Nfs1, whereas 4Fe–4S clusters seem to form through a reductive coupling mechanism only after detachment of IscU/Isu from the enzyme and its consequent dimerization.

The three-dimensional structures of several IscU/Isu orthologues have been solved both by NMR and X-ray crystallography [5,9–13]. They show different grades of compactness. The crystal structures, both of free and IscS/Nfs1-bound IscU/Isu, have a compact fold that consists of two α-helices sandwiched between a three-stranded anti-parallel β-sheet and three short α-helices [5,11,12]. The protein is more flexible in solution depending on the presence of a zinc cation which stabilizes the fold [9,10,13]. While the functional relevance of this cation is still unclear, we have recently shown that zinc does not interfere with Fe–S cluster formation and that IscU binds IscS as a fully folded structure [14,15].

The cluster bound to IscU is highly labile especially under aerobic conditions, as expected for a transient acceptor which readily delivers the cluster to more stable hosts [16,17]. Cluster coordination remains a matter of debate. It seems to involve three highly conserved cysteines (C37, C63, and C106 in *Escherichia coli* IscU) [18], although the role of C37 was questioned [19]. The fourth ligand could be the nearby H105 as supported by a recent radiolabeling study [20]. However, the crystal structure of IscS bound to cluster-loaded IscU_D39A from *Archaeoglobus fulgidus* shows a cysteine from IscS as a fourth ligand of the 2Fe–2S cluster [21]. While interesting, this observation cannot explain the coordination when IscU detaches from IscS, how the cluster can be formed chemically in vitro rather than enzymatically in the absence of the desulfurase [19], or why 4Fe–4S can be only formed on IscU/Isu after detachment of the protein from IscS/Nfs1 and formation of a dimer [7,8].

To complicate the matter, mutation of an aspartate to alanine (D39A in *E. coli* IscU) close to the coordination center stabilizes the cluster making it more persistent also under aerobic conditions, as observed for IscU/Isu variants from *Azotobacter vinelandii* [22], *Aquifex aeolicus* [23], *Schizosaccharomyces pombe* [16] and *Homo sapiens* [17]. The effect was

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* Corresponding author. Tel.: +44 20 8816 2630; fax: +44 20 8906 4477.
E-mail address: apastor@nimr.mrc.ac.uk (A. Pastore).

http://dx.doi.org/10.1016/j.bbamcr.2014.10.023
0167-4889/© 2014 Published by Elsevier B.V.

Please cite this article as: M. Adrover, et al., Anatomy of an iron-sulfur cluster scaffold protein: Understanding the determinants of [2Fe–2S] cluster stability on IscU, Biochim. Biophys. Acta (2014), http://dx.doi.org/10.1016/j.bbamcr.2014.10.023

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tentatively attributed to a decrease in solvent accessibility of the cluster [24,25] or to a stabilization of the folded state required for cluster-loaded complex formation [13]. However, since very little is known about the determinants of cluster coordination on IscU/Isu, it is difficult to rationalize these observations. A detailed study of IscU/Isu cluster coordination both in the wild-type protein and in the mutant is thus essential for a better understanding of the driving forces that yield cluster stabilization.

For this challenging endeavor, we have used a multidisciplinary approach involving a combination of multilayered quantum and molecular mechanical (QM/MM) calculations, together with different experimental studies. Among the computational methods, the ONIOM approach [26] has emerged as a powerful tool that allows analysis of even large proteins by using quantum mechanics treatment of the most interesting regions (e.g. the active site) but at the same time taking into account the environment at a classical molecular mechanical level. Intrinsic electronic properties can be derived from these calculations by the density functional theory (DFT). The DFT and ONIOM (QM/MM) methodologies have extensively been used to study metalloproteins [27].

We have used this approach to carry out a comparative study of wild-type IscU (IscU_wt) and its mutants (IscU_D39A and IscU_H105A) in their cluster loaded (holo) forms. We demonstrate, through the analysis of different independent parameters, that the instability of 2Fe–2S cluster bound IscU_wt can be explained by the conflicting role of D39 and H105 in the cluster coordination shell, which weakens the cluster protein interaction. This conflict is absent in the D39A and H105A mutants which, as a result, are thermodynamically more stable. We validated our computational conclusions using a combination of different spectroscopies including resonance Raman (RR), circular dichroism (CD), and UV–Vis absorbance. Our results were further comforted by a bioinformatic analysis that shows the high level of conservation of D39 and H105. Our study sets a new reference for understanding cluster formation and stability.

2. Materials and methods

2.1. Computational studies

The structure of *E. coli* IscU_wt was taken from the crystal structure of IscU in complex with IscS (pdb: 3LVL, chain A) [5], while the manual replacement of D39 to Ala provides the IscU_D39A model. The 2Fe–2S cluster was added to the resulting structures by superimposing them with the structure of IscU from *A. aeolicus* (pdb: 2Z7E, chain B). To demonstrate that the results are essentially independent from the initial choice we repeated the calculations using homology models obtained by the EXPASY server (http://swissmodel.expasy.org/) using the pdb structure 2Z7E as a template (Table 1 and Suppl. Mat.). The structures were relaxed at the MM level by the steepest-descent algorithm using the GROMACS package [28] and the GROMOS96 force field [29]. Hydrogens were added using the UCSF Chimera software (v.1.3), while H10 and H105 were protonated on $N_{\delta1}$ and unprotonated on $N_{\varepsilon2}$. A water solvation shell was added to the resulting structures using the TIP4PEWBOX solvation model.

Structures were prepared for QM/MM calculations by defining two layers (Fig. 1A). Experimental data indicate that the 2Fe–2S cluster is in a fully oxidized state when assembled on proteins [7,30]. We therefore assumed that the two Fe atoms are Fe^{3+} ions with a spin quantum number $S_i = 5/2$. Both spins are coupled anti-ferromagnetically and the total spin value for the cluster is $S = 0$. Geometry optimizations were carried out by the two-layer ONIOM(B3LYP/GENECP:UFF = QEQ) method implemented in the Gaussian 09 program [31]. The DFT-B3LYP functional was adopted for the higher level (QM layer) [32,33] in combination with the 6-311+G* basis set for H, C, N, S and O atoms, while the LANL2DZ [34] effective core potential was used to represent the core electrons of the iron atoms. Given that our system involves anti-ferromagnetically spin coupled interactions between the two high-spin irons, we used the broken symmetry (BS) approach for the QM region [35], a method that provides accurate results for the complex spin properties of Fe–S clusters [35,36]. The lower layer (MM level) was treated by the Universal Force Field (UFF) with charges derived using the charge equilibration (QEQ) scheme [37].

Truncated models were built from the optimized QM/MM structures by considering only the atoms included in the QM layer (Fig. 1B,C). The valence of the truncated carbon atoms was satisfied adding hydrogen atoms. The resulting structures were optimized at the BS-B3LYP/6-311++G** theory level freezing the positions of all heavy atoms. Afterwards, single point calculations were carried out using the same theory level and a density based solvation model (SMD) to simulate the effects of the water (ε 78.4) [38]. Natural bond orbital (NBO) analysis [39] was used to evaluate the NBO charges and determine the bond orders. Topological analysis of the computed wave functions at the SMD-BS-B3LYP/6-311++G** level was performed using the AIM2000 package [40] to quantify intra- and inter-molecular interactions.

Table 1
Geometric parameters of the 2Fe–2S cluster and of the atoms close to it. The distances are taken from the IscU_wt and IscU_D39A structures optimized at the detailed QM/MM computational level. Two starting structures were used: 3LVL and 2Z7E. The values obtained for the latter are in parentheses. Numbering is according to Fig. 1.

Interatomic distances (Å)	IscU_wt	IscU_D39A
Fe(1)–Fe(3)	2.892 (2.902)	2.788 (2.826)
Fe(1)–S(2)	2.288 (2.242)	2.280 (2.284)
S(2)–Fe(3)	2.300 (2.414)	2.218 (2.231)
Fe(3)–S(4)	2.347 (2.326)	2.235 (2.246)
S(4)–Fe(1)	2.248 (2.314)	2.278 (2.296)
Fe(1)–S(5)	2.446 (2.401)	2.392 (2.347)
Fe(1)–S(7)	2.236 (2.320)	2.219 (2.263)
Fe(3)–N(9)	2.688 (2.418)	2.147 (2.169)
Fe(3)–S(10)	2.397 (2.404)	2.301 (2.339)
Fe(3)–O(12)	1.991 (1.927)	–
Fe(3)–C(12)	–	4.500 (4.590)
Angles (*deg*)		
Fe(1)–S(2)–Fe(3)	78.1 (77.0)	76.6 (77.4)
Fe(1)–S(4)–Fe(3)	77.9 (77.4)	76.3 (76.9)
S(7)–Fe(1)–S(5)	90.2 (89.6)	104.4 (103.0)
S(10)–Fe(3)–N(9)	81.2 (79.3)	104.4 (104.6)
O(12)–Fe(3)–S(10)	94.3 (89.1)	–
Dihedral angles (*deg*)		
Fe(1)–S(2)–Fe(3)–S(4)	−2.7 (2.4)	−16.7 (−8.1)
S(10)–N(9)–Fe(3)–Fe(1)	−178.5 (−167.7)	−175.8 (177.1)
Fe(3)–Fe(1)–S(7)–S(5)	163.5 (169.8)	162.0 (168.8)
S(2)–S(4)–Fe(3)–S(10)	−157.8 (−177.9)	141.4 (135.8)

2.2. Protein production

Recombinant *E. coli* IscS, IscU_wt and its D39A and H105A mutants were over-expressed in *E. coli* and purified as previously described [41,42]. In short, they were produced as fusion proteins with a His-tagged GST and purified by affinity chromatography using Ni-NTA agarose gel (QIAGEN). All purification steps were carried out in the presence of 20 mM β-mercaptoethanol. The collected proteins were cleaved overnight from GST by TEV protease and further purified by gel-filtration chromatography on a Superdex 75 26/60 column (GE Healthcare). Protein purity was checked by SDS-PAGE and by mass-spectrometry.

2.3. Circular dichroism (CD) measurements

Far-UV CD measurements were performed on a Jasco J-715 spectropolarimeter (Jasco UK Ltd, Great Dunmow, UK) equipped with a cell holder thermostated by a PTC-348 Peltier system. Far UV CD measurements were performed at 25 °C in 10 mM Tris–HCl buffer at pH 8 using protein concentrations of 7–35 μM. The spectra were recorded

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