New Engineered *Archaeoglobus fulgidus* Ferritin as Potential Supramolecular Biotransducer for Metals Detection

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Introduction

In this work, we have evaluated the potential use of ferritin protein from *Archaeoglobus fulgidus* (Af-FER) both wild type (wt) and mutated by introducing cysteine residues, as biotransducer for the realization of biosensors for metals detection. The first example of application of this kind of biosensors was related to characterize the binding capacity of the Af-FER internal cavity towards iron II ions by means of both SPR and electrochemical techniques.

Ferritin from *Archaeglobus fulgidus*: main characteristics and modifications

The Af-FER is a unique thermostable protein cage endowed with notable structural features that makes it an extremely versatile tool for engineering nanodiagnostics. The protein is made of 24 subunits, symmetrically assembled in a spherical shape as indicated in figure 1. Unlike all other ferritins, the protein is able to dissociate under low salt conditions and reassociate in the presence of common salts, at a concentration higher than 0,2 M. Moreover, once reassociated to form the spherical ferritin molecule, Af-FER displays large triangular openings on the surface that delimit channels of about 40 Å length, i.e. about 10-fold wider than the common iron entry channels in classical ferritins.

Mapping Internal Cavity Residue (blue external, red internal)

The internal cavity of the wt protein has been mutated in order to insert a cysteine residue (figure 2) for possible specific functionalization (mutant QC). According to the scheme below, Q164 residue in the E-helix, near the C-terminus of the protein, has been mutated to cysteine. The mutated protein QC thus contains 24 cysteines pointing towards the interior of the cavity for appropriate functionalization. The importance of the internal functionalization is immediately apparent, in that it leaves the external surface of the molecule completely free for further coupling. Af-FER thus offers an unique possibility of creating a water soluble cage structure with openings for ligand entry.

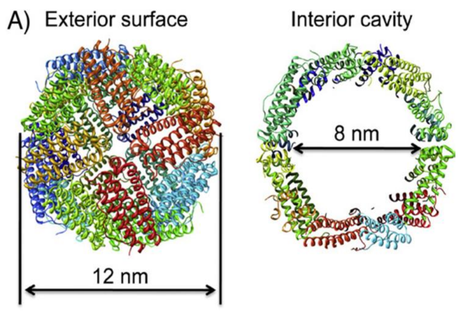


Figure 1. Classical ferritin assembly. Each of the 24 subunits assembles within a 4-fold symmetry axis thus delimiting a 12 nm protein cage.

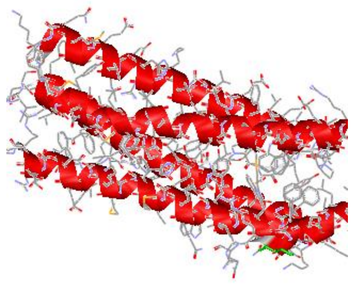


Figure 2. Single subunit of Af-FER. The relevant residue in position 164 is depicted in green.

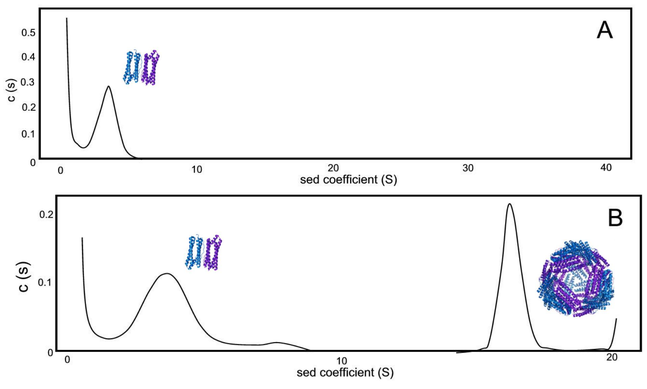


Figure 3. A: Analytical ultracentrifugation of the mutant QC in the presence of Hepes buffer 25 mM pH 7. 100% of the protein is in dimeric form (s = 3.5)

B: Analytical ultracentrifugation of the mutant QC in the presence of NaCl 0.2 M. 50% of the protein is in dimeric form (s = 3.5) and 38% in the form of 24-monomers (s = 16.3). At NaCl 0.4 M all the protein was in the form of 24-monomers .

SPR Experiments

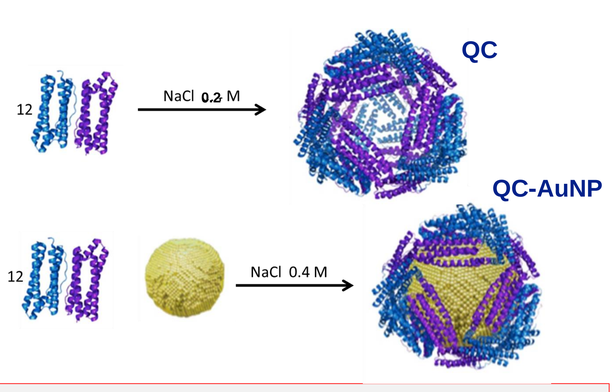


Figure 4. The mutant QC was dissociated at low salt concentration (< 0.2M NaCl). The nanoparticles (AuNPs) were added in defect with respect to the protein. Then the ionic strength was increased with 0.4 M NaCl, allowing the structure C to reassemble as described in the figure.

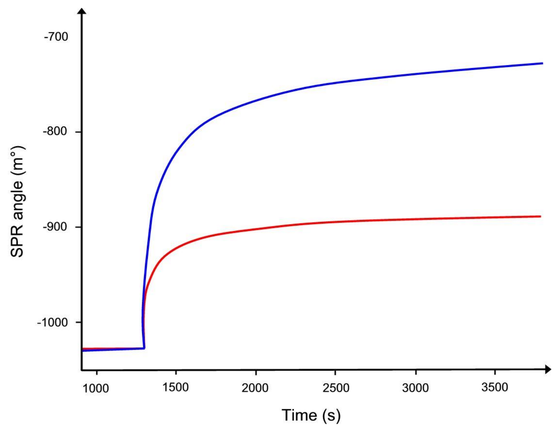


Figure 5. SPR sensorgram shows the irreversible immobilization of mutant QC (red line) and of QC-AuNP (blue line) on SAM-functionalized surface.

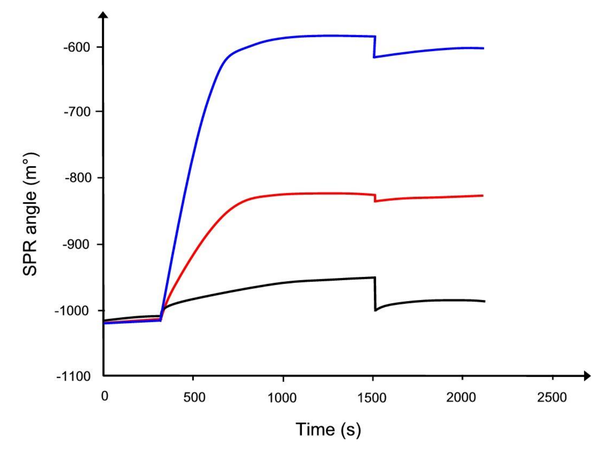


Figure 6. SPR sensorgrams obtained after blank subtraction for the detection of the affinity interaction between Af-FER, immobilized on SAM-modified surface and variable concentration of Iron (II) (mM): 2 (black line), 5 (red line), 9 (blue line). All measurements were performed in 25 mM HEPES+NaCl 0.2M buffer solution (pH=7.5) at 25 °C.

Electrochemical Experiments

All the electrochemical experiments were performed by adsorbing the protein onto Multi-walled carbon nanotubes screen printed electrodes (MWCNTs-SPE) from DropSens in HEPES 25mM+NaCl 0.2M buffer solution pH=7.5 at 25°C. Figure 7 is referred to the Fe(II) oxidation at the ferroxidase site of the protein in the presence of oxygen, this step allows the binding of ferrous iron and dioxygen to the ferroxidase site. The resulting electrode has been washed and characterized as showed in Figure 8, bringing in evidence of the binding of the metal onto the protein structure.
From the cyclic voltammetric (CV) behaviour has been possible to calculate the amount of iron loading as 1750 Fe/protein cage.

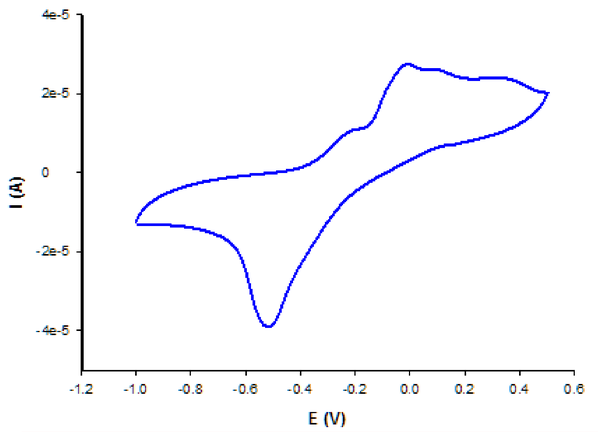


Figure 7. CV, measured using a solution of Fe (II) 50 mM at scan rate of 10 mV/s, in oxygen atmosphere.

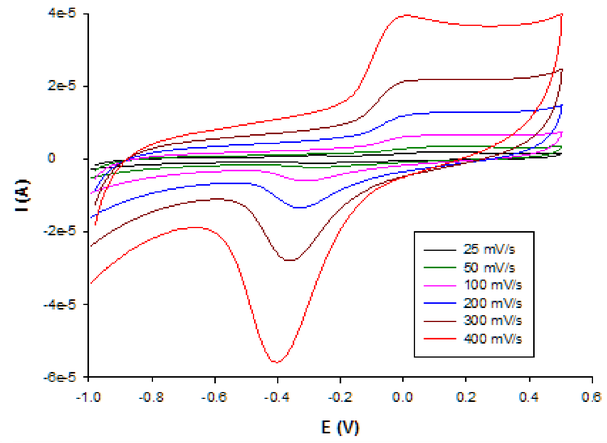
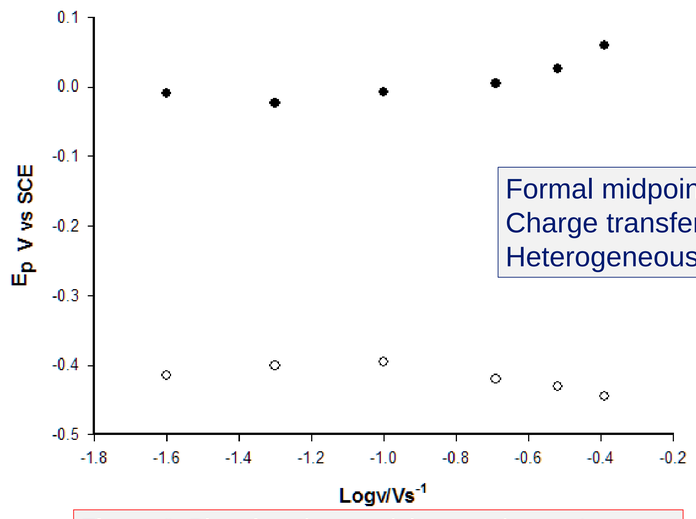


Figure 8. CVs, measured using a solution of Fe (II) 50 mM at different scan rates, in nitrogen atmosphere.



Formal midpoint potential: -0.20 V
Charge transfer coefficient: 0.69
Heterogeneous Electron transfer rate constant: 7.81 s^{-1}

Figure 9. Plot of peak potential separation vs. Log v.

Conclusions

In this work, we have produced and characterized ferritin from *Archaeglobus fulgidus* (Af-FER). To evaluate its capacity to bind divalent metals, in particular iron, SPR and electrochemical experiments have been realized. These experiments bring evidence of its capacity to bind iron II, which, at concentration higher than 20mM remains inside the protein structure as evidenced from the voltammetric experiments. In order to enhance and extend the affinity of this protein towards metals ions we are engineering the internal cavity of Af-FER by inserting several residues. In particular we have realized a ferritin mutant (QC mutant) by replacing glutamine in cysteine residues into the internal cavity. The QC mutant has been employed to perform the encapsulation of gold nanoparticles (5 nm diameter) within the cavity of ferritin by utilizing the hyperthermophile ferritin's tendency to reversibly disassemble at low salt concentrations. The SPR experiments realized comparing the SPR signal arising from the immobilization of QC mutant and QC-AuNP mutant onto the SAM functionalized disk surface confirm the encapsulation of AuNPs into the protein structure which could be useful for applications involving surface plasmon resonance.

The possibility to perform appropriate functionalization of the internal surface thus provides an unique tool for analytical, catalytic or diagnostic purpose.