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Photo-oxidation of tyrosine in a bio-engineered bacterioferritin 'reaction centre'—A protein model for artificial photosynthesis ☆

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ABSTRACT

The photosynthetic reaction centre (RC) is central to the conversion of solar energy into chemical energy and is a model for bio-mimetic engineering approaches to this end. We describe bio-engineering of a Photosystem II (PSII) RC inspired peptide model, building on our earlier studies. A *non-photosynthetic* haem containing bacterioferritin (BFR) from *Escherichia coli* that expresses as a homodimer was used as a protein scaffold, incorporating redox-active cofactors mimicking those of PSII. Desirable properties include: a di-nuclear metal binding site which provides ligands for bivalent metals, a hydrophobic pocket at the dimer interface which can bind a photosensitive porphyrin and presence of tyrosine residues proximal to the bound cofactors, which can be utilised as efficient electron-tunnelling intermediates.

Light-induced electron transfer from proximal tyrosine residues to the photo-oxidised $ZnCe_6^{\bullet+}$, in the modified BFR reconstituted with both $ZnCe_6$ and Mn^{II}, is presented. Three site-specific tyrosine variants (Y25F, Y58F and Y45F) were made to localise the redox-active tyrosine in the engineered system. The results indicate that: presence of bound Mn^{II} is necessary to observe tyrosine oxidation in all BFR variants; Y45 the most important tyrosine as an immediate electron donor to the oxidised $ZnCe_6^{\bullet+}$ and that Y25 and Y58 are both redox-active in this system, but appear to function interchangebaly. High-resolution (2.1 Å) crystal structures of the tyrosine variants show that there are no mutation-induced effects on the overall 3-D structure of the protein. Small effects are observed in the Y45F variant. Here, the BFR-RC represents a protein model for artificial photosynthesis.

1. Introduction

Photosynthetic organisms (plants, algae and cyanobacteria) are nature's own sophisticated solar panels and carbon sequestering systems. Oxygenic photosynthesis, a process that converts solar energy into chemical energy today sustains a broad variety of complex higher life forms on this planet, through oxygen evolution and carbon fixation. The component regarded as the 'engine-room' of this process is the Photosystem II (PSII) reaction centre (RC). The RC chemically drives unfavourable reactions, by extracting energy from photons [1,2]. All natural reaction centres function in essentially the same way, with only minor variations in their cofactors. Minimally, a reaction centre holds a photoactive pigment (P) and an acceptor molecule (A). Upon illumination, the excited state P^* can interact with a nearby acceptor and undergo electron transfer to form a charge-separated state (P^+/A^-) [1]. The molecular organisation of a reaction centre is essentially a series of transmembrane protein regions that localise the P^+/A^- pair to span the membrane. The core structure of all reaction centres is C_2 symmetric and in some systems the symmetry leads to bifurcated electron transfer pathways [1].

To assemble artificial reaction centres on protein scaffolds, the two minimal requirements are (i) selection and incorporation of pigments/chromophores for light capture, that initiate photochemistry and (ii) the choice and placement of redox-active intermediates for electron transfer. Electron donors and acceptors perform primary charge separation and their properties underpin the efficiency of the system. Detailed principles determining design and engineering of light capture and electron transfer processes between electron donor and acceptor molecules through redox-active intermediates in both natural and *de novo* protein scaffolds have been discussed previously; see [3–5].

We have used *Escherichia coli* bacterioferritin (BFR) as a protein scaffold to introduce key redox active intermediates in a PSII reaction

☆ This paper is dedicated to the memory of Warwick Hillier, who saw this work to completion, however tragically passed away after protracted illness, just prior to the submission of this paper. He was a dear friend and colleague to us all, who will miss him dearly, as will we feel the broader science community.

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centre inspired *in vitro* model system [4,6]. BFR is a soluble non-toxic iron storage and detoxification protein [7,8] that assembles into a dodecamer (24-subunit) with a hollow sphere of ~8 nm internal diameter (Fig. 1a). The BFR monomeric subunit is a four helix bundle (~18.5 kDa), where the four helices align anti-parallel to each other with a small fifth helix at the C-terminus, which aligns almost perpendicular to the rest of the bundle (Fig. 1b) [9,10]. Each of the four-helix bundles contains a di-nuclear metal binding site that binds two Fe^{II} atoms with histidine (H54 and H130) and glutamate (E51, E18, E127 and E94) residues as capping ligands. The two atoms are connected by the two bridging carboxylate groups (E51 and E127) (Fig. 2a). Single methionine residues, one from each monomer, provide a pair of axial ligands to the metal centre of iron-protoporphyrin IX (haem group) in the assembled BFR homodimer (Fig. 2b).This haem binding site at the dimer interface of the two four helix bundles is hydrophobic [9].

The BFR homodimer was used to reverse engineer PSII RC reactions, providing a novel way to explore the complex pathways of light-driven electron transfer in an artificial PSII analogue system. Extending from our earlier work [6,11], we here demonstrate (i) binding of $Mn^{II,II}$ at the di-nuclear metal binding site, (ii) binding of a photoactive pigment ($ZnCe_6$) at the hydrophobic pocket of the protein, by replacing the intrinsic haem group [12] and (iii) photo-oxidation and localisation of tyrosine residue (s) in the engineered BFR system.

In previous work, light-induced experiments in the modified BFR suggested that, upon photo-activation of the bound $ZnCe_6$, the weakly coupled $Mn_2^{II,II}$ at the di-nuclear site is oxidised and a ~ 25 G wide EPR spectrum centred at $g = 2.0058$ is observed [6]. This ~25 G wide spectrum was assigned to a tyrosine radical because of its line shape and the absence of quinone electron acceptors as possible contributing radicals [13,14]. The source of this spectrum was not established.

There are seven tyrosine residues per BFR monomer and therefore fourteen possible tyrosine residues per homodimer that might be redox-active. The three BFR tyrosine residues Y25, Y58 and Y45, which are within electron tunnelling distance from both the photoactive pigment and the metal centre, were sequentially mutated to phenylalanine (Fig. 3). Tyrosine 25 which is 4.3 Å from the nearest metal is highly conserved amongst other ferritins and bacterioferritins [15]. It is also hydrogen bonded to Glu 94, which is one of the metal ligands. Tyrosine 58 is unique to *E. coli* BFR in its position and is commonly replaced by a leucine or a phenylalanine residue in other ferritins. Tyrosine 45 provides van der Waals contact to the haem within the hydrophobic pocket at a distance of 3.1 Å [9].

Site-directed mutagenesis of tyrosine to phenylalanine has previously been used to identify the tyrosine radicals $Y_D^{\bullet}$ and $Y_Z^{\bullet}$ in PSII [13,16–18]. Here, we have constructed three tyrosine variants of the engineered BFR, resolved their crystal structures (~2.1 Å) and present a highly probable photo-oxidation electron transfer pathway within the fully assembled (all cofactors bound) BFR reaction centre.

2. Materials and methods

2.1. Molecular methods

In this study BFR^1 was used as the control. BFR^1 is a double mutant of the wild type *E. coli* BFR with two external histidine residues replaced by arginine residues (H46R and H112R) [6,11]. This is because, in other studies, spontaneous ligation of $ZnCe_6$ to histidine residues in both *de novo* peptides and to cytochrome b562 has been reported [19,20]. The three tyrosine variants (Y25F, Y45F and Y58F) were generated using the *bfr*^{1} gene (cloned in pET30 Xa/LIC vector) as the template. This was done using the Quick Change Site Directed Mutagenesis Kit (Strategene). All genes were sub-cloned in frame with a N-terminal 6 x histidine tagged ubiquitin sequence (H_6 -Ub), in a fusion *E. coli* expression system pHUE vector for protein expression [21,22]. The sub-cloning of the relevant genes was a two-step process. First, all genes were PCR amplified to introduce a 5′- *Sac*II and a 3′- *Hind*III cloning site and cloned into pGEM®-Teasy cloning vector (Promega) by TA cloning. The PCR primers used were *Sac*II 5′-TCCGCGGTGGAGATATGAA AGGTGATACTAAAG-3′ and *Hind*III 5′-AGATCCGCGAAGAAGGTTAAGCTT-3′. Correctly transformed plasmids were identified by blue–white selection. Second, sequenced mutant *bfr*^{1} genes were cloned into the *Sac*II-*Hind*III cut pHUE using standard ligation protocols.

2.2. Protein expression and purification

BFR^1 and the tyrosine variants were over-expressed in *E. coli* BL21 (DE3) cell line, where protein expression was induced using 1 mM isopropyl-β-D thiogalactopyranoside (IPTG). The recombinant his-tagged protein (H_6-Ub-BFR) was purified using Immobilised Metal Affinity Chromatography (IMAC)-nickel-sepharose chromatography

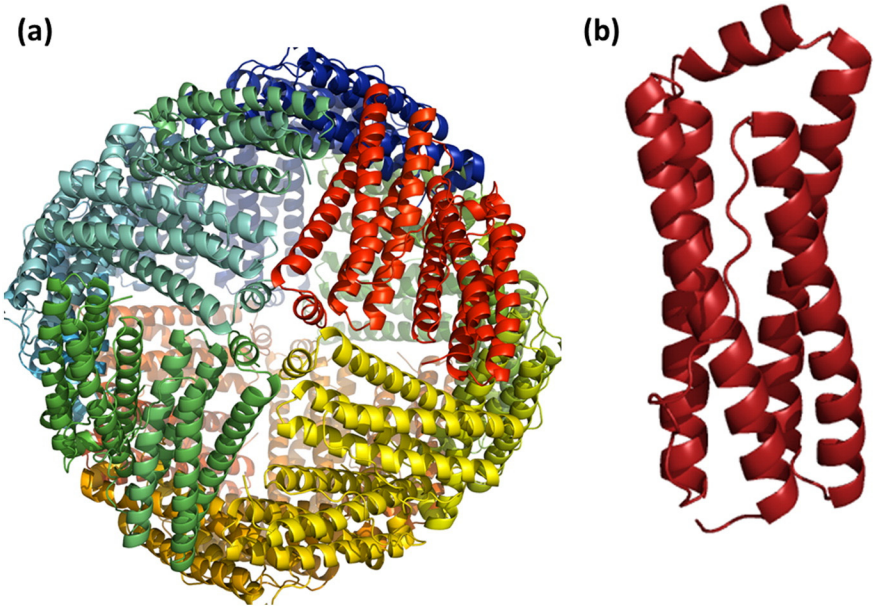

Fig. 1. Cartoon diagram of *E. coli* bacterioferritin (BFR). (a) Spherical, dodecameric (24-subunit) form of BFR; (b) monomeric subunit (four-helix bundle) of BFR (PDB file: 1BCF). Figure taken from Hingorani and Hillier 2012 [4].