

**Main Manuscript for**

Identification of a redox switch by X-ray photoreduction in a cyanobacterial FutA iron binding protein

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Main Text

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Table 1

**Abstract (220 of 250)**

Marine cyanobacteria can utilise an ABC transporter for iron uptake, which requires a membrane bound transporter and a periplasmatic FutA protein to bind iron in the ferric state (Fe3+). However, in the cytosol FutA proteins are known to bind ferrous iron (Fe2+). We studied the cyanobacterium *Prochlorococcus*, where a single FutA protein must perform both functions. While crystallographic studies of metal binding proteins are hampered by X-ray induced photoreduction, in this case leading to conversion from ferric to ferrous oxidation state, we instead used serial femtosecond crystallography (SFX) and neutron diffraction to obtain crystallographic structures of the oxidised state, revealing penta-fold coordination of the metal. X-ray induced photoreduction gave access to the reduced ferrous state by low-dose data collection on an X-ray home source, revealing a prominent structural change with a repositioned Arg203 side chain to balance charge changes in the iron binding site. To resolve this transition, a dose/time series was collected using serial synchrotron radiation crystallography (SSX). Structural changes were mapped at several dose points up to 110 kGy before global radiation damage becomes too severe, defining the sweet spot of such analyses in this case. The structural plasticity observed allows *Prochlorococcus* FutA to bind both, ferric and ferrous, and may reflect an adaptation mechanism for bacteria where genome reduction has resulted in loss of specialised FutA proteins.

**Significance Statement (109 of 120)**

Oceanic primary production by marine cyanobacteria is a main contributor to carbon and nitrogen fixation on earth. *Prochlorococcus* is the most abundant photosynthetic organism on Earth, responsible for a comparable carbon fixation to the net global primary production from agriculture. The bacterium’s remarkable ecological success is based on its ability to thrive in low nutrient waters, but growth is directly limited by iron availability. The FutA protein is required for iron binding, uptake and homeostasis. Our study uses X-ray photoinduced iron reduction at room temperature to study how FutA binds ferric (Fe3+) or ferrous iron (Fe2+). The accompanying structural changes characterize a structural switch in the redox active protein.

**Main Text**

**Introduction**

Iron is the fourth most abundant element in the Earth’s crust (1). However, because of its poor solubility, primary production in large oceanic and freshwater environments is limited by iron uptake (2, 3). In oxygenated aqueous environments, iron predominantly exists in the ferric (Fe3+) state with a solubility of 10-18 M (4). Ferric iron precipitates to form ferric oxyhydroxides (5) thought not to be generally bioavailable (6). Marine phytoplankton require iron in the photosynthetic electron transport chain (7) and in the nitrogenase enzyme (8, 9); thus, iron availability directly limits photosynthesis (10) and nitrogen fixation (11).

Cyanobacteria of the *Prochlorococcus* genus are able to fix four gigatons of carbon per annum, which is comparable to the net primary production of global agriculture (12). *Prochlorococcus* bacteria dominate bacterial populations in tropical and subtropical oligotrophic ocean regions (13). One of the factors for ecological success is the exceptional ability of this bacterium to thrive in low nutrient waters (14). Adaptation includes reduction in size to 0.5 – 0.7 m, and *Prochlorococcus* are believed not only to be the most abundant but also the smallest photosynthetic organisms on Earth (15, 16). Reduction in size maximizes area-to-volume ratio for metabolic efficiency, but the tradeoff is an extensive genome reduction; thus, *Prochlorococcus* also bears with 1.6-2.7 Mb the smallest genome known for any free-living phototroph (17).

Cyanobacteria may harbor multiple iron uptake systems, owing to the vital importance of iron for bacterial survival (9, 18, 19). Common is the use of iron chelates (siderophores), organic ligands used to solubilize iron (20, 21), where uptake typically involves the TonB transport system (22). The majority of the *Prochlorococcus* species lack genes for siderophore biosynthesis (23, 24); instead, the bacterium primarily relies on the Fut ABC transporter system for iron uptake (25). Here, specialized iron binding proteins sequester elemental iron and deliver it to ABC transporters for uptake (26). The Fut system typically contains two homologous substrate binding proteins (SBPs): FutA2 has been assigned a periplasmic ferric iron binding protein (27, 28), whereas the functional assignment of FutA1 remains unclear; FutA1 has been shown to bind ferric iron (27, 29), but can localize to the reducing environment of the cytoplasm, where it must bind ferrous iron and has a function to protect the photosystem against oxidative stress (30-32). The *Prochlorococcus* Fut uptake system is economized by reduction to one FutA protein, posing the question of whether the single *Prochlorococcus* FutA can bind both iron species. Furthermore, we have previously shown that the single FutA protein of the marine cyanobacterium *Trichodesmium* may indeed have dual localization and function (33), suggesting a single FutA protein can bind both iron species.

We wanted to understand how the *Prochlorococcus* FutA protein would structurally encode plasticity, enabling it to bind both iron species. This study needed to consider the effects of specific radiation damage (34) that would lead to photoreduction of ferric iron to ferrous iron resulting from the X-rays used in such crystallographic studies. We determined the *quasi* zero-dose structure of FutA in the Fe3+ bound state using XFEL serial femtosecond crystallography. Neutron diffraction not only avoided X-ray induced photoreduction but also gave the protonation states of chelating amino acids. The ferrous state was observed using X-ray induced photoreduction and was collected on an X-ray home source. Structural differences observed were mapped with a dose/time series that was collected using serial synchrotron radiation crystallography (SSX) and a fixed target chip setup, where we observed an optimal dose scenario before global radiation damage becomes dominant and outcompeted the ability to visualise structural change.

**Results**

**The ferric iron state of FutA.** In this study, the FutA protein from the marine cyanobacterium *Prochlorococcus* was expressed in recombinant cell culture, using an *E. coli* expression system. Overexpression leads to protein being produced in insoluble form. Inclusion bodies were harvested, and protein was refolded using rapid dilution into a buffer at pH 9.0, containing L-Arginine and ammonium iron (III) sulphate. Purified protein was burgundy red.

The Electron Paramagnetic Resonance spectroscopy (EPR) spectrum taken in solution shows a sharp signal at about 150 mT (equal to a g-value of 4.3 at 9.4 GHz) and several weaker signals between 80 – 140 mT, **Fig. 1a**. A spectroscopic index (g-value) of 4.3 is indicative of a penta-coordinated high-spin Fe3+ ion bound to FutA. The weaker signals can be related to high order spin transitions of ferric iron. Treatment with ten times excess of sodium dithionite caused the loss of the EPR signal, **Fig. 1a**, which is explained either by reduction of ferric iron to EPR-silent ferrous iron, or by loss of the metal.

To determine structure of FutA in the Fe3+ state, we used a serial femtosecond crystallography approach (SFX) to avoid changes to the iron centre through X-ray induced photoreduction, **Table 1**. These experiments were carried out at an X-ray free electron laser source (XFEL) where short high-intensity X-ray pulses deliver diffraction patterns that are collected before the sample is destroyed (35). While XFEL experiments generally can induce X-ray radiation damage changes (36, 37), it has been shown that damage-free data can be collected as long as sufficiently short pulses (< 20 fs) are used (38). Hence the pulse length used in this study was 10 fs and is taken to provide a *quasi* zero-dose structure of FutA, free from the effects of specific radiation damage and thus preserving the ferric state.

To carry out these experiments we optimised crystallisation. Starting from macroscopic crystals we changed the crystal conditions to obtain microcrystal slurries suitable for SFX, as described previously (39).FutA crystal slurries had to be prepared directly before the experiment to avoid ageing effects. Suitable preparations contained crystals that averaged approximately to 20 x 7 x 7 m3 in size. We used a fixed-target silicon nitride chip system to deliver to the X-ray interaction region, synchronized with the XFEL pulses (40). Care was taken to optimise data quality by attenuating the XFEL pulse in order to make best use of the dynamic range of the detector and minimise overloads. Taking a minimal multiplicity of ten as a guide, which is normally achieved with about ten thousand indexed patterns, the final dataset was merged from data obtained from three chips, using 150 l of microcrystal slurry each.

The overall structure of FutA has a classic ABC transporter substrate binding protein fold, with two domains hinged over a substrate-binding cleft, **Fig. 1b**. In *Prochlorococcus* MED4 FutA, the N-terminal domain is comprised of residues 1-98 and 232-280, while the C-terminal domain is made up from residues 99-231 and 281-314, coloured magenta and blue in **Fig. 1b**, respectively. SBPs can be classified based on their overall fold and their substrate binding site (41, 42). The overall fold of FutA is characterised by two domains connected by two short hinges, classifying *Prochlorococcus* MED4 FutA as “D type”.

The iron-binding site in the substrate-binding cleft is open to the surrounding solvent. Coordinating the iron, four tyrosinates are identified in FutA, namely Tyr13 from the N-terminal as well as Tyr143, Tyr199 and Tyr200 from the C-terminal domain, **Fig. 1c**. The binding site geometry is trigonal bipyramidal, where Tyr13, Tyr143 and Tyr200 form the trigonal plane with iron at its centre. The axial ligands are Tyr199 and a water molecule. The conservation of these key tyrosine amino acids in the substrate binding site classifies FutA as a Class IV SBP (43).

To confirm the presence of tyrosinates in FutA we determined the crystallographic structure of FutA by neutron diffraction to 2.1 Å resolution, **Table 1**. Protonation states of amino acids are revealed from the neutron Fobs – Fcalc omit map, where positive density indicates sites of successful hydrogen-deuterium exchange. The lack of deuterium on the iron coordinating Tyr13, Tyr143, Tyr199 and Tyr200 suggests these residues are indeed tyrosinates, contributing overall four negative charges to the iron centre, **Fig. 1d**. In contrast, the side chain of Arg103 is observed to be fully protonated, providing a single positive charge.

**Characterisation of X-ray induced photoreduction of ferric FutA.** RedOx active proteins present an experimental challenge to X-ray crystallography as changes in the oxidation state are induced by the incident X-ray beam. Whilst every X-ray structure suffers from phenomena of both, global and specific radiation damage (44), the latter is a particular problem for proteins with electron avid and thus RedOx active centres. Transition metals are particularly sensitive to specific radiation damage(34, 45), and changes resulting from X-ray induced photoreduction have been documented for doses as low as 33 kGy(38, 46, 47).

Upon X-ray exposure, the burgundy red crystals lose colour. To quantify this effect, we employed *in crystallo* UV-Vis spectroscopy. We carried out these experiments at cryogenic temperatures to avoid effects of re-oxidation through air. On incident X-ray irradiation, there are notable changes in the region of the ferric iron maximum (max = 438 nm) and in the region of the solvate electron peak (max ~ 600 nm), **Fig S1a**. To characterise photoreduction of the iron centre and relate iron reduction to accumulated radiation dose, the absorbance at 438 nm was plotted against dose, as shown in **Fig S1b**. The curve plateaus at about 120 kGy; it did not change further after one hour of X-ray irradiation, equivalent to a dose of 314 kGy (not shown).

We then calculated the percentage loss of absorbance at max = 438nm. Under the assumption that the bleaching observed represents the kinetics for photoreduction of ferric to ferrous accurately, the experiment suggests that at about 10 kGy a photoreduction to a level of 75 % occurs. Indeed, changes manifested immediately after the experiment and started at doses lower than 1 kGy. Moreover, the increase in absorbance at 600 nm due to solvated electrons is likely to affect the absorbance measurements at 438 nm. Thus, the decay at 438 nm is presumed to be more severe than measured, demonstrating the exceptional sensitivity of the ferric iron to X-ray induced photoreduction.

**Structural transitions between ferric and ferrous states of FutA.** Serial crystallography methods are uniquely suited for low dose investigations at ambient temperatures as radiation dose is spread across thousands of crystals. They can additionally help to understand transitions in biomolecules as functional motions are not “frozen out”. To map the effects of X-ray induced photoreduction of the iron centre, we used a fixed target approach and synchrotron radiation to obtain a defined dose slicing at room temperature. The experimental set-up has been described by us previously (48).

Two dose-series with ten dose points each were collected, one with 5 kGy and one with 22 kGy dose-slicing, **Table S1**. Isomorphous difference density maps (Fon – Fo1) were produced using the *RIDL* (49) pipeline which uses SCALEit (50) to scale the differences in observed structure factors (i.e. Fon – Fo1). SCALEit details the isomorphous difference between two datasets (Diso) which is defined as the difference in structure factor amplitudes (FPH – FP). An increase in the Diso value with resolution indicates two datasets are non-isomorphous. The SFX ferric FutA structure could not be used as a zero-dose reference for the serial data since it was non-isomorphous, as indicated by the Diso metric (not shown). Instead, the first dose point at 5 kGy was used as reference to produce the isomorphous difference density maps for both series. After a total dose of 110 kGy, the Diso metric indicated datasets were no longer isomorphous, as evidenced by the increase in B-factors and the decrease in I/I, see **Table S1**. This observation can be explained by the accumulation of global radiation damage, and as a result, isomorphous difference density maps above 110 kGy do not clearly visualise structural changes. All structures of dose points were individually refined, and the increase in B-factor in consecutive doses was addressed by B-factor sharpening.

Inspection of isomorphous difference density maps indicate loss of density around the coordinating water and around Tyr13 on increasing dose of 50 kGy and 88 kGy, **Fig. 2a**. Additionally, the (Fon – Fo1) difference density maps reveal positive difference density located close to the guanidine group arginine 203, **Fig. 2b**. The electron density is readily explained when a second conformation of the arginine side chain is fitted at a dose of 88 kGy, **Fig. 2c**. As indicated in the metrics (Diso, increase in B-factors, decrease in I/I, see **Table S1**), significant global damage is observed at higher doses. This leads to disappearance of distinct features, in the difference maps at higher doses. Similar loss of specific radiation damage has been observed previously (51), and is likely a phenomenon isolated to room temperature data collection as specific and global radiation damage are much less decoupled (51, 52).

**The photo-reduced ferrous iron state of FutA.** Owing to the high sensitivity of the iron centre to X-ray irradiation, we wanted to use this effect in the study of the ferrous Fe2+ state of FutA protein. Like all other studies, we performed the experiment at ambient temperature not to restrict any structural changes that may accompany photoreduction. We used a large (0.5 mm) crystal mounted in a sealed capillary and collected data at a home source, **Table 1**. The home source X-ray rotating anode generator at the Cu *K* wavelength of 1.54 Å (8.04 keV) exposed the single crystal of FutA for approximately 1 hour to obtain a full dataset to 1.7 Å, which equated to a total dose of 110 kGy.

The iron chelating water was clearly resolved in electron density, in contrast to the SSX dose series in the photoreduced state. This indicates that the ferrous iron is penta-coordinated, similar to what was seen in the ferric binding state. This data collection scenario resolved the alternative conformation of arginine 203 even more clearly, which was refined as the single conformation in this case, **Figure 3a**.

The Arg203 sidechain was highly ordered in the home-source structure and overlays with the difference density observed at a dose of 88 kGy, **Fig. 3b**. In contrast, the electron density for the Arg203 sidechain is highly disordered in the ferric state SFX structure, and the side chain was fitted in a different rotameric structure, **Fig. 3c**. The alternative conformation of Arg203 brings the guanidino group within 5 Å of the Tyr13 and Tyr200 oxygens in the photoreduced state. Distances of 4.8 Å between the η1 amide of Arg203 and the alkoxy group of Tyr200, and with 4.6 Å between the η2 amide of Arg203 and the alkoxy group of Tyr13 were observed.

The positive charge of the arginine sidechain contributes an additional positive charge to the iron binding site. As photoreduction is expected to change the redox state from ferric Fe3+ to ferrous Fe2+ iron, Arg203 would neutralise the binding site to give it a net zero charge, see Table in **Figure 3**. The positioning of the arginine side chain towards the binding site may therefore be a hallmark of the ferrous state, which is assumed to have been generated during the experiment through X-ray induced photoreduction.

**Discussion**

The adaptation of the marine cyanobacterium *Prochlorococcus marinus* is a remarkable story of ecological success, making this photosynthetic organism the most abundant on earth. Two factors are particular important, the ability to survive under limiting nutrient conditions, and size reduction, and both of these factors put evolutionary pressure on the iron uptake system of the bacterium. As a result, a single gene encodes for the FutA protein. For organisms that have a single gene, dual localisation of FutA was shown (33), posing the challenge of both ferric and ferrous, for which FutA from *Prochlorococcus* provides a role model and that allowed the organism to survive with a single gene when at the same time under pressure for reduction in genome size(14, 25). Our study of the FutA protein was driven by the question how these proteins could bind either, Fe3+ and Fe2+.

The structures of FutA presented here allow us to derive a plausible mechanism for two binding states of iron. If this is compared to FutA proteins from other organism, in particular the well-studied iron binding proteins FutA1 and FutA2 from *Synechocystis* FutA (28, 29, 53, 54), then two specialized proteins serve iron binders for the ferrous (FutA1) and ferric states (FutA2). Consequently, in *Synechocystis* FutA2 is localized to the oxidative periplasm, where one expects a ferric binding protein, while FutA1 is cytosolic, and under the reducing conditions of the cytosol binds ferrous. Previously we showed that the single FutA protein in the cyanobacterium *Trichodesmium* may indeed have dual localization and function (33), and this may be similarly the case for *Prochlorococcus* FutA.

Commonly, structure determination using X-ray crystallography is complicated by X-ray induced radiation damage. Of all protein structural features susceptible to specific radiation damage, metal centres are known to be one of the most sensitive, with metal ions with higher oxidation numbers rapidly photoreduced by X-ray induced photoelectrons (38, 46, 47). For Fe3+, we show here that this happens within the first few kGy of irradiation, with a reduction to 75% of the original iron signature peak occurring at 10 kGy (**Figure S1**).

Making an enemy a friend, we have developed SSX protocols to use X-ray induced damage for specific photoreduction of the metal center, this trapping the ferrous state at room temperature. We reasoned that data collection at ambient temperatures would allow for protein flexibility (55), and the SSX method enabled us to follow the transition of Fe3+ to Fe2+ during the experiment. Indeed, in the structure determined a positively charged amino acid side chain (arginine 203) is either “in” or “out”, characterizing ferrous and ferric states.

To understand the transitions involve, we used an SSX approach and constructed a time/dose series. We determined the “sweet spot” of data collection would be exposure to less than a dose of about 110 kGy, using the criterium of isomorphousness of crystals. We detected a decrease in I/σI between the 22 kGy and 220 kGy from 9.47 to 2.88 for all reflections and from 3.04 to 0.63 for high resolution reflections, **Table S2**. Global radiation damage in the experiments shown has correlated with loss of specific radiation damage. The loss of high-resolution diffraction signal would result in a reduction in high-resolution structural information and a decrease in the difference density. The result was that difference density features “wane” at higher doses, even from refined structures. This problem was less severe in the home source dataset. In addition, the longer data collection time used here (about one hour) compared to the 120 ms data collection time of the SSX dataset may have come to the advantage, allowing any displaced water to replenish.

Determination of the ferric state by serial femtosecond crystallography was complemented by neutron diffraction studies that, by nature, do not cause photoreduction. Does the neutron structure agree with the zero dose serial femtosecond crystallography structure? If so, I would find this worth mentioning here. The neutron diffraction studies show protonation states, revealing that the four amino acid side chains of Tyr13, Tyr143, Tyr 199 and Tyr200 were deprotonated, coordinating the iron as tyrosinates. A water molecule visible in the zero-dose structure of the ferric state and in the low dose structure of the ferrous state takes the fifths position in a penta-fold coordination sphere. As we show with the SSX dose series, this water is displaced with accumulating dose. Positive charges are contributed to the binding site by Arg103 in the ferric state and by Arg103 and Arg203 in the ferrous state, making the binding site next-zero, **Figure 3d**.

Conclusion: The protein structures with iron bound in different oxidation states help explain how the intrinsic structural plasticity of FutA accommodate ferrous as well as ferric iron species. Translated into a molecular mechanism, an arginine side chain flip provides a charge balance. We have used photoreduction as an advantage to study this transition of ferric and ferrous binding. The acute sensitivity of FutA to specific radiation damage illustrates the requirement for dose limiting data collection regimes for metal binding SBDs. To our knowledge the SFX FutA structures presented in this paper are the first radiation damage free structures, representing the ferric iron binding SBP state.

**Materials and Methods**

*Molecular biology.* *Prochlorococcus* MED4 *futA* excluding the region encoding the signal peptide (UniProt ID: Q7V0T9, amino acids 27 – 340, as predicted by *SignalP*(56)) was cloned into pET-24b(+) using the NdeI / HindIII restriction sites.

*Protein purification.* *Escherichia coli* Bl21 (DE3) cells (NEB) were transformed and cultured in 1 L of lysogeny broth containing 50 g ml−1 kanamycin in 3 L baffled flasks in a shaker at 130 RPM and 37 °C. When the cell culture reached an OD600 of 0.4 the temperature was reduced to 18 °C. Protein expression was induced at an OD600 of between 0.6 – 0.8 by addition of IPTG to a final concentration of 1 mM. Cells were incubated for a further 20 hrs at 18 °C, before harvesting by centrifugation at 4000 x g (Avanti Jxn-26, JLA-8.1000 rotor). Bacterial cells (2-4g) were resuspended in 25 ml IBB buffer (0.1 M Tris buffered at pH 9, containing 0.5 M NaCl, 1% Triton-X, 5 mM MgCl2 and 10 mM β-mercaptoethanol) and lysed by incubation with 2 mg ml-1 lysozyme for 30 min and sonication for total pulse time of 150 seconds (Q700 Sonicator, 10 second pulse duration with 20 seconds between pulses). Centrifugation (40 mins, 125 000 x g, 4 °C, Optima XPN-80, Type 70 Ti rotor) yielded a pellet containing inclusion bodies that were washed by resuspension in IBB containing 2 M urea and harvested by a second centrifugation step as above. The pellet was solubilised by incubation in 200 mM Tris buffered at pH 9, 6 M urea, 10 mM -mercaptoethanol for 1 hr at 4 °C, before removing cellular debris by centrifugation as above. The solute contained FutA that was refolded by rapid dilution using a syringe into 2 L of 0.2 M Tris buffered at pH 9.0, containing 0.2 M NaCl, 0.4 M L-Arginine and 0.1 mM NH4Fe(SO4)2. After incubation at 4 °C for 48 h the protein was concentrated to 150 ml using an Amicon Stirred Cell (10,000 Da Ultrafiltration Disk, Merck), and subsequently dialysed for 24 hr at 4 °C against 2 L dialysis buffer (100 mM Tris buffered at pH 9.0, containing 145 mM NaCl). After dialysis, the protein was applied to a 5 ml HiTrap SP XL column (GE Healthcare) equilibrated with dialysis buffer. The protein was step-eluted with 0.1 M Tris buffered at pH 9.0, containing 320 mM NaCl. The final purification step by size-exclusion chromatography used a HiLoad 16/60 Superdex 200 column (GE Healthcare) equilibrated in 50 mM Tris buffered at pH 9.0, containing 300 mM NaCl. Absorbance at 280 nm was used to determine the fractions in which monomeric FutA eluted. FutA was concentrated using a Vivaspin 20 Centrifugal Concentrator, MWCO 10,000 Da (Sartorius) and stored at 4 °C.

*Removal of iron from FutA*. FutA was diluted to 0.5 mg ml-1 with neutralising buffer (100 mM Tris bufferd at pH 7, containing 300 mM NaCl), to a volume of 50 ml. Three successive rounds of dialysis against 500 ml 50 mM MES buffered at pH 6, containing 10 mM 2,2-dipyridyl and 100 mM sodium dithionite were carried out under anaerobic conditions, each for 1 hr at 21 °C. A final round of dialysis was carried out against 1 L neutralising buffer for 20 hr at 21 °C.

*Electron paramagnetic resonance.* FutA at a concentration of 50 M was placed in EPR quartz tubes and shock-frozen in liquid helium for data collection. Reduction of FutA was carried out by addition of 500 M sodium dithionite. X-band continuous wave EPR spectra were recorded on a Bruker eleXsys E500 spectrometer using a standard rectangular Bruker EPR cavity (ER4102T) equipped with an Oxford helium cryostat (ESR900). The spectrometer worked at X-band frequency with a 10 Gauss modulation amplitude and 2 mW microwave power. All spectra were recorded at 5 – 6 K.

*Protein Crystallisation.* Depending on experiment, FutA was crystallised by vapour diffusion, batch or seeded batch. The different crystallisation set-ups yielded crystals in space group P21 with varying sizes but related unit-cell parameters.

For vapour diffusion crystallisation, ~50 mg ml-1 FutA was crystallised in 24-well XRL plates (Molecular Dimensions) containing 0.2 M sodium thiocyanate, 10 - 35 % (w/v) PEG 3350 FutA and precipitant were mixed at a 1:1 ratio to a total volume of 2 l and crystallisation plates were incubated at 21 °C. Crystals appeared within 1 day and were typically 10 – 200 m in the longest dimension.

For batch crystallisation, ~50 mg ml-1 FutA was crystallised in a microcentrifuge tube containing 0.2 M sodium thiocyanate, 10 - 24 % (w/v) PEG 3350. FutA was mixed at a 1:1 ratio with precipitant to a total volume of 20 l and incubated at 21 °C. Crystals appeared within 3 days and were typically 200 – 1500 m in the longest dimension.

For seeded batch crystallisation, a seed stock was made by pooling 10 l of FutA vapour diffusion droplets containing crystals and adding 40 l 20% PEG 3350; this solution was then transferred onto Hampton Seed Bead and vortexed for 180 s. The seed stock frozen at 4 °C in 5 l aliquots for storage. Immediately prior to crystallisation the seed stock was thawed and diluted 1:100 with 0.2 M sodium thiocyanate, 20 % (w/v) PEG 3350. For crystallisation, ~50 mg ml-1 FutA was mixed with the diluted seeds and with 0.2 M sodium thiocyanate, 10 - 20 % (w/v) PEG 3350, at a ratio of 1:1.5:1.5 to a total volume of 150 l. The crystallisation solution was incubated at 21 °C and crystals appeared within 30 minutes. Crystals were typically 10 – 20 m in the longest dimension (39).

*Sample preparation for serial crystallography.* Crystals produced by seeded batch crystallisation were loaded onto silicon fixed-target ‘chips’, as described previously(38). Each chip accommodates 25,600 apertures with aperture sizes ranging from 4-20 m. For the experiments described here 150 l of FutA microcrystal slurry was loaded onto a glow-discharged chip containing 7 or 12 m sized apertures within a humidity-controlled chamber. Excess liquid was removed by applying vacuum, and the chip was sealed between two sheets of 6 m thick Mylar.

*RT serial synchrotron crystallography (SSX).* SSX data were collected at beamline I24, Diamond Light Source. Silicon nitride chips with 12 μm aperture size were used. After chip alignment, either single images (low dose data collection) or multiple images (dose series) were collected at each aperture. The radiation dose corresponding to a single X-ray exposure was calculated using *RADDOSE-3D*(57). For the dose-series, images were separated into doses for individual processing(48). Images were indexed and integrated using dials.stills\_process and scaled using cxi-merge (*DIALS* v2.0)(58).

*RT serial femtosecond crystallography (SFX).* SFX data were collected in a helium-filled flight tube to minimise air scattering using the MPCCD detector at SACLA, Japan, beamline BL2 EH3. The XFEL was operated at an X-ray energy of 11.0 keV using a pulse length of 10 fs with a repetition rate of 30 Hz. Synchronising chip translation with the XFEL pulse, data collection of each chip took roughly 12 mins. Images were stored in a hdf5 stream, applying indexing and pre-filtering for diffraction hits with *Cheetah*(59). For data processing, diffraction hits were indexed and integrated with *DIALS* (v3.0)(58) using the module *dials.stills\_process*. An image mask was generated manually using *dials.image\_viewer* to remove the beam stop shadow and Si diffraction spots arising from the chips. Data from a total of 3 and 2 chips were collected for Fe and apo FutA respectively. Integrated patterns were scaled and merged using the *DIALS* module *cctbx.xfel.merge*(60) to yield the final datasets at 1.6 Å and 1.7 Å resolution for Fe and apo FutA, respectively.

*RT Neutron crystallography.* For hydrogen-deuterium exchange, FutA crystals with a volume larger than 0.2 mm3 grown from batch crystallisation were transferred into crystal mother liquor prepared with deuterated water. Incubation at 21 °C for 24 hrs was followed by a second exchange and further incubation for 24 hrs. Crystals were then mounted in 1 mm sealed quartz capillaries. Data collection was undertaken at the BIODIFF instrument at Forschungsreaktor München II (FRM II), Germany, using a monochromatic neutron beam with a wavelength of 3.1 Å (60A). Of multiple crystals tested, two were selected for final data collection. Diffraction data were indexed and integrated using *HKL2000*(61) and the data from the two crystals were scaled and merged using *SCALEPACK*(61) to yield the final dataset at 2.1 Å resolution.

*RT home source crystal structure.* Data collected from a single crystal measuring 0.32 x 0.53 x 0.54 mm3 grown from batch crystallisation and mounted in a 0.7 mm sealed quartz capillary were collected at room temperature on a Rigaku 007 HF (High Flux) diffractometer equipped with the HyPix 6000HE detector (Southampton Diffraction Centre). The X-ray beam was collimated at 200 mm2 with a flux of 2.5 x 109 at 8.1 keV. The total exposure time of 1 hrs equates to a total dose of 64 kGy, as calculated using *RADDOSE-3D*(57). Data were integrated with *XDS*(62), and merged and scaled using *POINTLESS*(63) and *AIMLESS*(64).

*Structure determination and refinement.* Molecular replacement with *MOLREP*(65) used the *Synechocystis* PCC 6803 FutA2 as search model (PDB: 2PT1). *COOT*(66) was used for model building; refinement was carried out with *REFMAC*(67) for X-ray and with *PHENIX*(68) for neutron data. Coordinates have been deposited with the PDB under accession numbers …

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**Figures and Tables**

Diagram

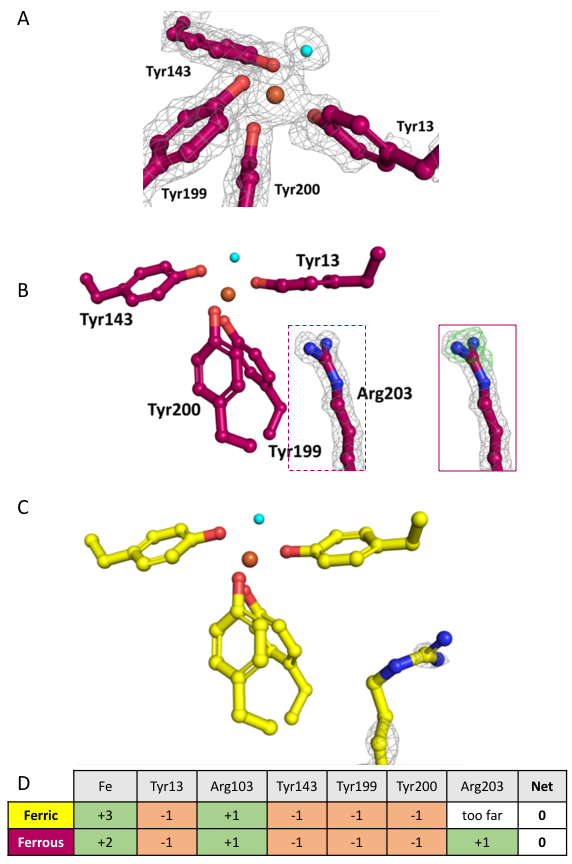
Description automatically generated

**Figure 1. Characterisation of the ferric Fe3+ state of FutA from *Prochlorococcus marinus* MED4.** **(a)** EPR spectrum for the iron complex (left) and for a chemically reduced sample (right). **(b)** FutA has a bi-lobal structure shown here as cartoon with the N-terminal domain shown in magenta and the C-terminal domain in blue. Amino acid side chains contributing to iron binding are shown in stick representation in yellow and red for carbon and oxygen atoms, respectively. Iron is shown in orange, and a coordinating water molecule is shown in light blue. **(c)** Ligand coordination shows the iron in a trigonal bipyramidal geometry. SFX structure determined by serial crystallography using an X-Fel source; color coding as in (a), grey dashes indicate the bonds and coordination sphere. **(d)** Iron binding site seen in the crystal structure determined by neutron diffraction. The positive electron density (green mesh, 3) indicates sites that have undergone hydrogen-deuterium exchange, confirming the change of tyrosinates. Carbons are shown in yellow for tyrosinates and light blue for Arg103, oxygen in red, nitrogen blue, iron as orange and the coordinating water as light blue spheres.

Diagram

Description automatically generated

**Figure 2.** Refined SSX structures from a dose series at RT. (a) 2Fo-Fc electron density (grey, contoured to 1.5 σ) and (b) Fo-Fc difference density (green / red, contoured at +/- 3 ). Shown are refined structures at radiation doses as indicated. (c) The difference density at 88 kGy dose allows fitting a second rotameric positioning of Arg203.



**Figure 3.** Comparison of thephotoreduced ferrous state and the ferric state of FutA. (a) The low dose home source structure (110 kGy) of FutA determined to 1.7 Å from a home source shows pentafold coordination of the iron. (b) The grey electron density around Arg203 is the refined 2Fo-Fc map displayed at 1.5 . Inset: the green map is an overlay with the Fo-Fc difference density map from the refined 88 kGy SSX data, displayed at 3 . (c) The quasi zero-dose SFX structure (yellow) shows weak density around Arg203, indicating disorder. (d) The table indicates the charge balance in the iron binding site between zero dose SFX and and photoreduced states. Assuming an overall net zero, this suggests ferric Fe3+ and ferrous Fe2+ binding, respectively.

**Table 1.** Data collection and refinement statistics for FutA structures reported (see **Table S1** for full listing).

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Data collection statistics | | | | | |  | | | | | | |  | | | | |
| **Data Collection** | | **SFX** | | | | **Neutron** | | **Home Source** | | | **SSX 5 kGy** | | | | |
| Temperature (°C) | | 21 | | | | 21 | | 21 | | | 21 | | | | |
| X-ray Energy (keV) | | 11.0 | | | |  | | 8.0 | | | 12.8 | | | | |
| # Integrated Frames | | 78743 | | | |  | |  | | | 5278 | | | | |
| # Merged Frames | | 78035 | | | |  | |  | | | 5170 | | | | |
| Space Group | | P21 | | | | P21 | | P21 | | | P21 | | | | |
| Unit Cell (a, b, c) | | 39.1, 78.3, 47.4 | | | | 39.5, 78.3, 47.9 | | 39.4, 78.0, 48.0 | | | 39.7, 78.7, 48.4 | | | | |
| b angle | | 97.9 | | | | 97.4 | | 98.2 | | | 97.8 | | | | |
| Resolution (all, Å) | | 30.10 – 1.60 | | | | 24.94 – 2.1 | | 47.55 – 1.70 | | | 40.97 – 1.76 | | | | |
| Resolution (HR, Å) | | 1.63 – 1.60 | | | | 2.18 – 2.1 | | 1.73 – 1.70 | | | 1.79 – 1.76 | | | | |
| Rsplit / Rpim1,2 | | 7.5 (12.6) | | | | 10.6 (33.4) | | 0.9 (8.5) | | | 23.5 (68.4) | | | | |
| CC **½** (%)1 | | 99.6 (90.6) | | | | 99.1 (45.4) | | 100.0 (98.6) | | | 92.3 (43.1) | | | | |
| 1 | | 8.6 (3.7) | | | | 4.6 (1.9) | | 71.7 (11.6) | | | 3.14 (0.41) | | | | |
| Completeness (%)1 | | 99.8 (100.0) | | | | 80.4 (48.1) | | 97.9 (83.5) | | | 100.0 (100.0) | | | | |
| Multiplicity1 | | 307.6 (132.7) | | | | 1.9 (1.1) | | 65.4 (42.1) | | | 26.2 (19.1) | | | | |
| Unique Reflections1 | | 37,259 (1,877) | | | | 13,748 (886) | | 30,881 (1,369) | | | 29,256 (1,467) | | | | |
| Wilson B-factor (Å2) | | 12.09 | | | | 6.88 | | 14.10 | | | 24.25 | | | | |
| Refinement statistics | | |  | | | | | | |  | | | | |
| **Data Collection** | | **SFX** | | **Neutron** | | | **Home Source** | | | | | **SSX 5 kGy** | | | | |
| Resolution (Å) | | 30.10 – 1.60 | | 24.94 – 2.1 | | | 47.55 – 1.70 | | | | | 40.97 – 1.76 | | | | |
| Rwork/Rfree | | 0.200 / 0.221 | | 0.238 / 0.263 | | | 0.159 / 0.189 | | | | | 0.188 / 0.237 | | | | |
| # Reflections all/free | | 37,259 /1919 | | 13,747 / 688 | | | 30847 / 1,588 | | | | | 29256 / 1503 | | | | |
| Number of Atoms | |  | | |  | | | |  | | | | |  | | | | |
|  | Protein | 2485 | | | 5592 | | | | 2539 | | | | | 2511 | | | | |
|  | Ion | 1 | | | 1 | | | | 1 | | | | | 1 | | | | |
|  | Water | 86 | | | 258 | | | | 121 | | | | | 179 | | | | |
| Ramachandran | |  | | |  | | | |  | | | | |  | | | | |
|  | Preferred | 304 | | | 307 | | | | 303 | | | | | 300 | | | | |
|  | Allowed | 5 | | | 2 | | | | 5 | | | | | 9 | | | | |
|  | Outliers | 1 | | | 1 | | | | 2 | | | | | 1 | | | | |
| B-factors (Å2) | |  | | |  | | | |  | | | | |  | | | | |
|  | Protein | 17.83 | | | 16.43 | | | | 19.52 | | | | | 33.32 | | | | |
|  | Ion | 7.57 | | | 6.27 | | | | 11.77 | | | | | 24.72 | | | | |
|  | Water | 23.32 | | | 24.19 | | | | 26.00 | | | | | 48.19 | | | | |
| R.M.S Deviations | |  | | |  | | | |  | | | | |  | | | | |
|  | Bond Lengths (Å) | 0.0158 | | | 0.012 | | | | 0.0100 | | | | | 0.0060 | | | | |
|  | Bond Angles (°) | 1.79 | | | 1.12 | | | | 1.98 | | | | | 1.35 | | | | |

1High resolution statistics in parentheses