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Time lapse, anomalous X-ray diffraction shows how Fe^{2+} substrate ions move through ferritin protein nanocages to oxidoreductase sites.

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Synopsis A series of high-resolution crystal structures of *Rana catesbeiana* ferritin M, loaded with Fe^{2+} substrate at different metal ion diffusion times, show a sequence of iron binding sites that allow visualize a possible path of substrate diffusion to the catalytic center.

Abstract

Ferritin superfamily protein cages reversibly synthesize internal biominerals, $Fe_2O_3{\cdot}H_2O$. Fe^{2+} and O_2 (or H_2O_2) substrates bind at oxidoreductase sites in the cage, initiating biomineral synthesis to concentrate iron and prevent potentially toxic reactions products from $Fe^{2+}$and O_2or H_2O_2 chemistry. By freezing ferritin crystals of *Rana catesbeiana* ferritin M (RcMf) at different time intervals after exposure to a ferrous salt, we obtained a series of high-resolution, anomalous x-ray diffraction data leading to crystal structures that allow to directly observe ferrous ions entering, moving along and binding at enzyme sites in the protein cages. The ensemble of crystal structures, from both aerobic and anaerobic conditions, provides snapshots of iron substrate bound at different cage locations that vary with time. The observed, differential occupation of the two iron sites in the enzyme OS (with ligands: Glu23, Glu58; His61 and Glu103, respectively) and other iron binding sites (with ligands: Glu53, His54, Glu57, Glu136, Asp140), reflects the approach of the Fe^{2+} substrate and its progression before the enzymatic cycle: 2 Fe^{2+} + $O_2 \rightarrow$ Fe^{3+}-O-O-$Fe^{3+} \rightarrow$ Fe^{3+}-O(H)-Fe^{3+} and turnover. The crystal structures also revealed different Fe^{2+}coordination compounds bound to the ion channels located at the 3- and 4-fold symmetry axes of the cage.

1. Introduction

Ferritins are nanocage proteins that synthesize and concentrate iron as hydrated ferric oxide biomineral (up to 4500 iron ions) inside the large central cavity of the protein cage (Liu & Theil, 2005). The 24 subunit ferritins are characterized by having 432 (O, octahedral) point symmetry with three 4-fold, four 3-fold and six 2-fold axes. The ferritin polypeptide subunits fold is the 4-helix bundle motif (helices H1-H4), completed by a short, fifth helix at the C-terminus (H5), external to the bundle. Each ferritin subunit has enzyme (oxidoreductase) activity (H-type) except in animal ferritins which coproduce a catalytically inactive subunit (L type) that co-assembles with active subunits in H:L ratios that are tissue specific (Arosio *et al.*, 2009). Amphibians have a second catalytic subunit, called M (or H'), which shares 64% of sequence identity with the human H-subunit. Homopolymeric M-subunit ferritins are often used as a eukaryotic ferritin model.

In ferritin, Fe^{2+} is the substrate of the reaction that occurs at the oxidoreductase sites, and ferric species are the products. The nature of the iron species involved in the catalytic reaction mechanism has been identified by spectroscopy but the exact nature and the number of the metal ligands has remained elusive.

Oxidation of iron in eukaryotic ferritins monitored by iron spectroscopy (rapid freeze quench Mössbauer and XAS) proceeds through at least three different species, as observed in *Rana catesbeiana* ferritin M (RcMf) model (Hwang *et al.*, 2000). In step one, Fe^{2+} reacts with O_2 converting the diferrous, high-spin state iron substrate, with an Fe−Fe distance of 3.44 Å, to a peroxodiferric (DFP) intermediate, characterized by a very short Fe−Fe distance of 2.53 Å and with a typical UV-vis absorbance at 650 nm. DFP appears in less than 25 ms, and (step two) decays to diferric μ-oxo/hydroxo species with a Fe−Fe distance of 3.00 Å. It is the latter diferric oxy species that is proposed to translocate from the catalytic sites to the protein biomineralization cavity (step three) (Hwang *et al*, 2000).Several amino acids in the interior of the ferritin 4-helix bundles may act as metal ligands during the catalytic reaction. Initial attempts to identify ferritin protein ligands to iron using, as iron proxies, redox-inert, non-native, divalent metal cations, which would remain trapped at the active site, were complicated by the different coordination preferences of the various metal ions used (Ha *et al.*, 1999; Tosha *et al.*, 2010; Toussaint *et al.*, 2007). The lifetime of DFP species is too short for conventional x-ray structural characterization. However, kinetic x-ray crystallography can be used to characterize the iron binding sites in ferritin and their evolution with time.

In the first reported crystal structure of a vertebrate ferritin (RcMf) with iron bound, a binuclear iron cluster was found bound in each ferritin subunit oxidoreductase site (OS). The observed Fe-Fe distance of 3.1 ± 0.1 Å at 2.7 Å resolution, is consistent with the presence of a diferric oxo/hydroxo bridge. One of the Fe^{3+} ions was coordinated to monodentate Glu23, bridging Glu58 and His61, while the second Fe^{3+} was coordinated to bidentate Glu103 and bridging Glu58 (Bertini *et al.*, 2012). In another ferritin-iron structure, obtained after a shorter time of iron diffusion into the crystal, a Fe ion was bound to His54, which lies in the close proximity of the above residues. Such data suggest that Fe^{2+} binds at a site with His54, before enzymatic oxidation (Bertini *et al.*, 2012).

Here, we present a series of high-resolution, anomalous X-ray diffraction experiments on single protein crystals of RcMf treated with ferrous ions for different periods of time, under aerobic and anaerobic conditions. The results provide detailed information on the identity of the most populated, transient iron binding sites in the pathway from cage entry pores, along the protein cage to the OS, as well as direct analysis of Fe^{2+} binding at the OS itself.

2. Materials and methods

2.1. Mutagenesis

The plasmid pET3a carrying the RcMf gene was used for introducing H54Q mutation. The primer for mutation was designed with the web-based software Primer X (http://bioinformatics.org/primerx/). Site directed amino acid substitution in RcMf was generated by PCR, with the expression plasmid pET-3a RcMf DNA as the template, using the Quik Change II Site-Directed Mutagenesis kit from Agilent following the provided protocol. Gene sequencing was done to confirm the mutations (Primm Srl, Milan, Italy).

2.2. Recombinant Expression and Purification of *Rana Catesbeiana* M ferritin and variant.

RcMf was expressed and purified using adapted protocols from the literature (Turano *et al.*, 2010). *E. coli* BL21(DE3) pLysS cells containing the mutated plasmid DNA were cultured in rich media (Luria Broth) and grown at 37°C and 180 rpm until OD_{600} reached 0.7. The expression started after addition of IPTG (1 mM final concentration), and the culture was incubated for 4 h. After harvesting by centrifugation, the cells were lysed using lysozyme and sonication. In order to purify ferritin, coagulation of temperature-sensitive proteins and two chromatography steps were involved. First, the supernatant of the centrifuged lysate was heated at 65°C for 15 minutes and the aggregated proteins were sedimented by ultracentrifugation (40 min, 40000 rpm, 4°C). Then, the supernatant was dialyzed against 20 mM Tris buffer, pH 7.5. Finally, the sample was loaded onto a 120 mL SP Sepharose anion-exchange column (GE Healthcare, Sweden) and eluted with a linear NaCl gradient (0-1 M) in 20 mM Tris buffer, pH 7.5. Pertinent fractions were determined by SDS-PAGE, and those containing ferritin were concentrated using an Amicon ultracentrifugation filter device with a molecular weight cutoff of 14 kDa (Millipore, U.S.A.). The sample was then loaded onto a 120 mL Dextran size exclusion column and eluted with 20 mM Tris buffer, pH 7.5. Fractions were monitored by UV-vis spectroscopy and pooled together to obtain a final ferritin sample. Iron and other metal ions were removed by four dialysis steps at room temperature using 2 L each of 20mM Tris, pH 7.5, 5 mL 500 mM EDTA, pH 5.8, 10 mL ammonium thioglycolate, to reduce and chelate the iron, followed by four dialysis steps at room temperature using 2 L each of 20 mM Tris pH 7.5.

2.3. Stopped-flow measurements.

The kinetics of iron uptake by demetallated wt-RcMf, Mg-loaded wt-RcMf and demetallated RcMf-H54Q variant were monitored as the change in absorbance at 650 nm (diferric-peroxo) or at 350 nm ($[Fe^{3+}O]_n$ species) (Hwang *et al.*, 2000). Measurements were carried out using a UV/visible, stopped-flow spectrometer (model SX-18 MV, Applied Photophysics, UK). Absorbance curves were measured after mixing equal volumes of 100 µM protein, as subunits, in 200 mM MOPS, 200 mM NaCl, pH 7.0 buffer, with a freshly prepared solution of 200 µM ferrous sulfate in 1 mM HCl, in order to obtain a 2 Fe/subunit (or 48 Fe/nanocage) complex. To monitor the effect of Mg^{2+} on protein-dependent Fe^{2+} oxidation rates, under conditions comparable to those used in crystallization experiments, these experiments were repeated using a ferritin solution preincubated for 2 hours in 0.1 M of $MgCl_2$. Each measurement was performed 3 times. 4000 data points were collected during 10 s. Initial rates of diferric-peroxo and $[Fe^{3+}O]_n$ species formation were determined from the linear fitting of the linear portion of the absorbance curves recorded at 650 nm and 350 nm. The data are averages from a set of 3 experiments (see Supplementary Material and Supplementary Figures).

2.4. Protein crystallization.

Crystals of wt-RcMf and of the H54Q variant were grown under aerobic environment using the hanging drop vapor diffusion method. Drops were prepared by mixing equal volumes of demetallated protein solutions (7-14 mg/mL in 20 mM Tris HCl at pH 7.5) and precipitant solutions ranging from 1.6 M to 2 M $MgCl_2 \cdot 6H_2O$ and 0.1 M bicine buffer at pH 9.0. The final pH in the equilibrated drops is about 8.0. The low pH RcMf crystals were grown at pH 6.5 by using the same protein and precipitant solution in 0.1 M bis-tris propane buffer. Drops were equilibrated over a 600 µL precipitant solution at 4°C. Octahedral crystals of the wild-type protein and of the variant appeared in 2-5 days and completed growth in about 10 days. The crystals harvested for all the experiments were carefully chosen to have similar dimensions ranging between 0.12 mm to 0.15 mm as can be appreciated from the pictures shown in Figure S3. All crystals belong to the cubic space group F432 and contain one RcMf subunit per asymmetric unit.

The time controlled iron loading study was performed under aerobic conditions by allowing iron free diffusion from solid ammonium Fe^{2+} sulfate (Mohr's salt: $[(NH_4)_2Fe(SO_4)_2] \cdot 6H_2O$) to crystals of wt-RcMf and of the H54Q variant. Mohr's salt crystals were inserted directly in the crystallization drop containing the RcMf crystals. This methodology has been devised in view of the fact that our repeated attempts to use conventional soaking experiments of RcMf crystals in conditions analogous to those reported for *P. furiosus* (Tatur *et al.*, 2007) or pennate diatom (Marchetti *et al.*, 2009; Pfaffen *et al.*, 2013), never lead us to detect any iron ion bound to the protein.

After one, two, five, fifteen, thirty and sixty minutes for the wt-RcMf and one, fifteen and sixty minutes for the RcMf-H54Q variant, iron loading was stopped by flash freezing crystals in liquid nitrogen.

wt-RcMf crystals were also grown under anaerobic conditions (controlled nitrogen atmosphere in a glove box equipped with a catalyzer for dioxygen reduction) using the sitting drop vapor diffusion method. Drops were prepared by mixing equal volumes of a demetallated and

deoxygenated protein solution (7 mg/mL in 20 mM Tris HCl at pH 7.5) and deoxygenated precipitant solution of 1.6 M $MgCl_2$ and 0.1 M Bicine at pH 9.0. The final pH in the equilibrated drops is again about 8.0. Drops were equilibrated over 800 µL of a reservoir solution at 4°C. The crystals grew in 6-10 days in the same F432 cubic space group of aerobically grown crystals. Iron loading was performed in the anaerobic environment by the above procedure. However, the crystals suffered severe cracking for long exposure times to Mohr's salt, limiting iron diffusion to a maximum of three minutes.

2.5. X-ray data collection, structure solution and refinement.

X-ray crystallographic data were collected at the Elettra beamline XRD-1 (Trieste, Italy) equipped with a Pilatus 2M detector and at the ESRF (European Synchrotron Radiation Facility, Grenoble, France) beamlines BM14 and ID29 equipped with a MarMosaic 225 CCD and a Pilatus 6M-F detector, respectively. Data collection statistics are reported in Table 1. Data were integrated using the program MOSFLM 7.0.6 (Leslie, 2006) or XDS (Kabsch, 2010a; Kabsch, 2010b) and scaled with the program SCALA (Evans, 2006) from the CCP4 suite (Winn *et al.*, 2011).

Initial models were obtained by molecular replacement using the software MolRep (Vagin & Teplyakov, 1997) and using one subunit of the wt-RcMf as search model for the rotation and translation functions structure (PDB code: 3KA3), after excluding metal ions and water molecules. The positions of the iron ions were determined from the anomalous Fourier difference maps calculated using the program FFT from the CCP4 suite. The anomalous signals corresponding to iron ions ranged between 4.5 and 50 σ in the anomalous difference maps.

In all experiments we collected anomalous data using x-ray wavelengths short enough to obtain high quality data (little absorption), but where the anomalous signal of iron is still strong. When allowed by the synchrotron beamline characteristics, we also collected on the same crystal, full three-wavelength anomalous data: remote high energy, peak of the Fe K-edge and immediately below the Fe K-edge, to obtain the unambiguous proof that the observed anomalous signal belonged to iron and not to adventitious metals or other atoms (see Table 1).

The structures were refined using the program REFMAC5 (Murshudov *et al.*, 1997) from the CCP4 suite. The refinement protocol involved a sequence of iterative manual rebuilding of the model and maximum likelihood refinement. The molecular graphic software Coot (Emsley *et al.*, 2010) was used to visualize the structures and for the manual rebuilding and modeling of missing atoms into the electron density. Water molecules were added using the program ARP/wARP (Langer *et al.*, 2008). The final models were inspected manually and checked with the program Coot (Emsley *et al.*, 2010) and Procheck (Laskowski *et al.*, 1993). Structure solution and refinement statistics are reported in Table 2. All figures were generated using the program CCP4mg (McNicholas *et al.*, 2011). Occupancies of almost all the metal sites are fractional. Metal occupancies have been adjusted to obtain atomic displacement parameters comparable with the surrounding protein atoms. The water molecules bound to the metal ion refined at partial occupancy have been refined by using the same occupancy of the parent metal ion. The crystallographic evidence refers to a spatial average of the single RcMf subunit constituting the crystal asymmetric unit, over the

24 subunits composing the holoprotein. Each subunit is further averaged on the whole crystal. Partial occupancies observed for the iron ions and the different conformations of several amino acid side chains can be the result of either local disorder and/or of the space and time average of the metal binding events (differential diffusion in different portions of the crystal) occurring in the crystals before freezing.

2.6. PDB accession codes.

Final coordinates and structure factors of the RcMf obtained under aerobic conditions have been deposited in the Protein Data Bank (PDB) under the accession code 4LQH for the iron free RcMf and 4LPJ, 4LQJ, 4LYX, 4LYU, 4LQV, 4LQN, for RcMf loaded with iron for 1, 2, 5, 15, 30 and 60 minutes, respectively. Final coordinates and structure factors for RcMf obtained under anaerobic conditions and loaded with iron maintaining the oxygen-free environment, have been deposited in the PDB under the accession code 4MY7. Final coordinates and structure factors of the RcMf variant H54Q have been deposited in the PDB under the accession code 4MKU for the iron free state and 4ML5, 4MN9, 4MJY, for the iron loaded states determined at 1, 15 and 60 minutes, respectively.

3. Results

A series of structures that identified the intermediate Fe^{2+} binding sites in RcMf, from the entry channels to the OS, were obtained at high resolution. Crystals of wt-RcMf and of its H54Q variant grown at pH 8.0 using $MgCl_2$ as precipitant (see Methods section), were mixed with crystalline ferrous ammonium sulfate powder and frozen after 1, 2, 5, 15, 30, 60 minutes of free diffusion of the Fe^{2+} ions in the crystallization drop, in the presence of the second reaction substrate, dioxygen. The Fe^{2+} uptake by wt-RcMf was also studied under anaerobic conditions for comparison. The selected reaction conditions were such to sufficiently slow down the kinetics of Fe^{2+} movement along ferritin protein cages and reaction with dioxygen, thus allowing the observation of intermediate iron binding sites. Indeed, $[Mg(H_2O)_6]^{2+}$ ions inhibit iron mineralization in RcMf, by blocking formation of the diferric peroxide catalytic intermediate (Liu & Theil, 2004) as shown by stopped-flow kinetic measurements (reported as Supplementary Material). The structures of the iron-bound RcMf crystals obtained at different times were compared to the structure of iron-free RcMf. The Fe^{2+} oxidation state in the aerobic crystals has been attributed on the basis of the observed Fe-Fe distances and by comparison with the structure determined under anaerobic conditions and at different pH values (*vide infr*a).

The detailed description of the x-ray method used and of all crystal structures is reported as Supplementary Material.

3.1. Time zero structure of RcMf

The crystal structure of iron-free RcMf at atomic resolution (time zero structure) was used as the reference structure to appreciate the changes occurring in the protein upon iron binding. Figure 1, time 0 reports a view of the oxidoreductase site of RcMf occupied by three Mg^{2+} ions bound at the same locations previously observed in the RcMf structure crystallized under similar conditions

(PDB: 3KA3) (Tosha *et al.*, 2010). Two of these metal ions (Mg1 and Mg2 in Figure 1, time 0) are bound with partial occupancy to sites that are close, but not identical, to the iron binding sites seen in the iron-bound RcMf low resolution structures (Bertini *et al.*, 2012) (PDB codes: 3RBC, 3RGD). Stopped-flow solution measurements on Fe^{2+} uptake by RcMf in the presence of Mg^{2+} ion concentration comparable to that used for crystallization, provide experimental evidence of the slowing-down of the iron oxidation kinetics as reported in Supplementary.

3.2. One minute free diffusion structure of RcMf

The first Fe^{2+} binding site to be populated by iron in RcMf is the OS identified by Glu23, Glu58 and His61 and defined as Fe site 1 (Figure 1, time 1). Iron is bound to site 1 with an occupancy of 0.30. No other sites appear to be occupied by iron. On binding to site 1, Fe^{2+} causes only a small rearrangement of the side chains of the three ligands, while the three Mg^{2+} ions, which occupy the OS in the iron-free protein, remain in place.

The coordination geometry of $Fe^{2+}$is a quite regular square pyramid completed by two water molecules forming the basal plane with Glu23 and Glu58 carboxylate oxygens. This coordination geometry appears to be dictated by the presence of the Val106 side chain at about 4.0 Å from Fe site 1, which hinders the binding of a sixth ligand to Fe1. Val106 is conserved in catalytically active eukaryotic ferritins. Mononuclear Fe^{2+} enzymes that activate O_2 (e.g. intradiol dioxygenases) contain the so-called 2-His-1-carboxylate facial triad active site (Costas *et al.*, 2004), where the protein ligands are bound to a face of the Fe^{2+} coordination octahedron while the opposite face is occupied by three water molecules that provide the access site for O_2. In RcMf, Fe1 cannot adopt such coordination as the side chain of Val 106 fills up that part of the site, hampering O_2 access. This is probably the structural explanation of the observed inability of Fe1 in ferritin to react with O_2 in the absence of the second iron ion as demonstrated by the observed formation of a dinuclear peroxo intermediate (Pereira *et al.*, 1998) displaying a UV-vis absorption spectrum completely different from that of mononuclear Fe^{3+} oxo/hydroxo complexes (Cho *et al.*, 2011).

3.3. Two minutes free diffusion structure of RcMf

After 2 minutes of free diffusion, Fe^{2+} is now observed bound to the second iron site in the OS (Fe site 2: ligands Glu58 and Glu103; 0.30 occupancy) and the occupancy of site 1 has increased from 0.30, at 1 minute, to 0.50 at 2 minutes (Figure 1, time 2). When Fe^{2+} binds at site 2, two of the Mg^{2+} ions in the OS are displaced, while the third Mg^{2+} ion (Mg1 in Figure 1, times 0 and 1) observed at time 0 in the OS is unaffected (not shown in Figure 1, time 2 for clarity). The longer time needed to observe Fe^{2+} binding to site 2can be explained by the necessity to displace two Mg^{2+} ions from that part of the cavity. Once again this well correlates with the slowing down of the reaction rate observed in presence of Mg^{2+}. The presence of only two protein ligands for Fe^{2+} in site 2 suggests a lower metal affinity for this site, in analogy with the findings of isothermal

titration calorimetry (ITC) on human H ferritin (HuHf) (Bou-Abdallah *et al.*, 2002; Bou-Abdallah *et al.*, 2003; Honarmand *et al.*, 2012).

Two minutes of Fe^{2+} free diffusion in the crystal allows completion of the coordination sphere of the dinuclear iron cluster in the OS. The Fe1-Fe2 distance is 3.63 Å. Fe1 displays square pyramidal geometry, as in the 1 minute structure. The Fe2 ion is bound to Glu58 (bridging it to Fe1) and to Glu103 as symmetric bidentate ligand. Fe2 coordination is completed by a bridging water/hydroxide molecule (Wb) and by a terminal water molecule. The geometry of the Fe2 site is irregular, somewhat between a square pyramid and a trigonal bipyramid.

3.4. Fifteen minutes free diffusion structure of RcMf

Free diffusion of Fe^{2+} for 5 and 15 minutes does not change the binding of Fe^{2+} in the OS except for an increase the occupancy at the enzyme centers from 0.5 to *ca.* 0.7 (Figure 1 time 15).

Additionally, two new significant maxima, close to Fe2 (Figure 1 time 15), appear in the anomalous difference map, which are named Fe sites 3 and 4, respectively. The Fe3 ion (Figure 1 time 15) is at very short distance (2.48 Å) from Fe2, bound to the Nδ1 atom of His54 (site 3 in Figure1 all sites) and with an incomplete coordination sphere. Such observations indicate that Fe2 and Fe3 cannot be simultaneously bound.

The Fe3 and Fe4 sites appear to represent binding sites for Fe^{2+} substrate that is approaching the OS. Interestingly the positions of Fe1 and Fe2 sites match those of Co^{2+} and Cu^{2+} found in recently reported RcMf crystal structures (Bertini *et al.*, 2012; Tosha *et al.*, 2010), where they share the same ligands with the exception of His54 that binds both Co^{2+} and Cu^{2+} in site 2 and not Fe^{2+}. The fact that Co^{2+} (Tosha *et al.*, 2010) and Cu^{2+}(Bertini *et al.*, 2012) are inhibitors of the catalyzed reactions of Fe^{2+} with O_2 in ferritin might reflect the similarity of Co^{2+}, Cu^{2+} and Fe^{2+} binding at the OS, and the conserved inability of ferritin to oxidize $Co^{2+}$and Cu^{2+}in contrast to Fe^{2+}.

3.5. Thirty and sixty minutes free diffusion structures of RcMf

A clearer view of iron bound at the ferritin OS is provided by the crystal structure of RcMf obtained after 30 minutes of free diffusion of Fe^{2+} (Figure 1 time 30). The iron sites 1 and 2 are the only ones occupied by Fe^{2+} and the Fe-Fe distance is 3.54 Å. The coordination spheres of the two Fe ions are better defined, and retain square pyramidal and distorted trigonal bipyramidal geometry observed at shorter time intervals, but with much less disorder of the side chains of the residues involved in metal binding. The 60 minutes structure provides the same view of the OS. The occupancy of Fe sites 1 and 2 is still partial and decreases from 0.7 to about 0.5 for Site 1 and 0.3/0.5 for Site 2 (Table S1). The lower occupancy of Fe in the ferritin OS after a longer exposure time suggests that in the period of time between 15 and 30 minutes turnover occurs at both Fe1 and Fe2 sites and depletes sites 3 and 4 that appear again empty.

3.6. Sixty minutes free diffusion structure of RcMf at pH 6.5

Crystals of RcMf have been also obtained in the same crystal form at the lower pH of 6.5 (see Methods section), with respect to pH 8.0 of the previous structures. This experiment allowed us to check the influence of the crystallization pH on the oxidation state of iron bound in the OS, and to have a comparison with the trigonal, low resolution structure obtained from crystals grown in sodium formate at about neutral pH (PDB code 3RBC) (Bertini *et al.* 2012). The pH 6.5 structure was determined after 60 minutes of free diffusion of Fe^{2+}. Under these conditions, two iron ions bind (0.40 occupancy) to the same oxidoreductase sites 1 and 2, maintaining the same coordination geometry observed at pH 8.0 and bridged by a water species (most probably OH^-). However, the two iron ions are now closer, with a Fe-Fe distance of 3.15(5) Å which is the same as the Fe^{3+}-Fe^{3+} distance observed in the 3RBC (Bertini *et al.*, 2012) and is characteristic of diferric-μ-oxo/hydroxo model compounds (Kurtz, Jr., 1990; Tshuva & Lippard, 2004; Vincent *et al.*, 1990). Diferric-oxo/hydroxo complexes are products of ferritin enzyme activity (Hwang *et al.*, 2000) and this structure suggests that at pH 6.5, *in chrystallo,* Fe^{2+} has been oxidized by dioxygen at the ferritin enzyme centers and that, at this pH, the rate limiting step is the release of the diferric product of the reaction from the OS.

3.7. Fe-RcMf structure under anaerobic conditions.

To validate our attribution of the iron oxidation state in previous structures as Fe^{2+}, the structure of iron adduct of RcMf was obtained using crystals grown under strictly anaerobic conditions at pH 8.0 and treated, anaerobically with Mohr's salt, for about 3 minutes. The diffusion time was the maximum possible before cracking of the crystals occurs (see Methods section). Such observation emphasizes the osmotic pressure that builds up from Fe^{2+} diffusing into the crystal in absence of enzymatic turnover and mineral growth; Fe^{2+} accumulation at ferritin metal binding sites and at less specific iron binding sites leads to breaking of native, intermolecular interactions.

The anomalous difference Fourier maps obtained from data collected anaerobically at and below the Fe K-edge (7130 eV) clearly show the presence of four iron ions in the OS (Figure 2) that are located, within experimental error, at the same positions observed in the aerobic crystals after 15 minutes of treatment with Mohr's salt. However, in the anaerobic crystals the Fe1 site reaches almost full occupancy, while the Fe2 is still at 0.50 occupancy. The Fe1-Fe2 distance is 3.64 Å, comparable to the analogous distances in crystals formed aerobically at pH 8.0 (Table S2), supporting our assignment to Fe^{2+} as the iron species present in all crystals used for our kinetic crystallography experiments.

3.8. Effect of H54Q substitution near the OS of RcMf

The protein environment near enzyme sites can play an important role in directing substrate to the active site. In the case of ferritin metal ions have been observed bound near the active sites, such as to His54 in the RcMf structures presented here and in our previous work (Bertini *et al.*, 2012). Gln58 in the human H chain takes the structural position of RcMf His54. In order to investigate the effect of the substitution of His with Gln on iron binding, we have produced the RcMf-H54Q variant crystallized under the same conditions as wt-RcMf.

Crystal structures of RcMf-H54Q have been determined, in the presence of air, in the iron free state and after 1, 15 and 60 minutes of iron treatment.

The iron-free RcMf-H54Q structure reported in Figure 3, time 0, shows the same Mg^{2+} binding pattern observed in HuHf (PDB code: 3AJO) and different from that of wt-RcMf (Figure 1, time 0) as described in detail in the Supplementary Material section. These findings indicate that the H54Q mutation renders the OS of RcMf very similar to that of HuHf.

The OS of RcMf-H54Q treated for 1 minute with Mohr's salt is shown in Figure 3, time 1. As in the analogous wt-RcMf, only the oxidoreductase site 1 is occupied by iron (about 0.70 occupancy) and the oxidoreductase Fe2 site is empty. However, the Fe3 and Fe4 sites are now occupied by iron as observed in the 15 minutes and the O_2-free RcMf structures (see Figure 1, time 15 and Figure 2 for comparison). The mutated residue Gln54 binds Fe3. At variance with wt-RcMf, a new iron site (Fe5) is found (Figure 3, time 1), located further away.

The structures of the RcMf-H54Q variant obtained after 15 and 60 minutes of treatment with Mohr's salt, show a similar pattern of iron binding (Figures 3 times 15, 60). The oxidoreductase cavity is occupied by a cluster of four iron ions bridged by a series of protein and water/hydroxide ligands.

The scenario depicted by this structure is complicated by the partial occupancy of the sites. Indeed, while Fe1 occupancy refines to about 0.80, those of Fe2, Fe3, Fe4 and Fe5 range from 0.40 to 0.25 as do the water molecules bound to them. Furthermore, the side chains of three protein ligands of the cluster (Gln54, Glu57, and Asp140) are observed in double conformations. Therefore, the Fe2-Fe3-Fe4 cluster and Fe5 ion most probably do not represent a structure that occupies these sites in all RcMf-H54Q subunits, but rather indicate a superposition of different states that are due to a dynamic process of the iron interaction with the protein. The multiple conformations adopted by Glu53, Glu57 and Asp140 suggest that these residues handle the metal ions from one site to the next one in the path to the OS.

3.9. Iron sites located on the ferritin symmetry axes

Inspection of the anomalous difference maps at the three-fold pores of wt-RcMf and of RcMf-H54Q variant obtained after 60 minutes of treatment with Mohr's salt shows two anomalous peaks surrounded by six electron density maxima, indicating the presence of two iron ions having the regular octahedral structure of a Fe^{2+}-hexa-aquaion (Figure 4). The Fe^{2+}-aquaions are located

exactly on the ferritin cage 3-fold axis at different depths from the protein surface (about 11.0 Å and 18.0 Å from surface, respectively). They are engaging two series of six and nine symmetry-related H-bonds, respectively, to Asp127 and Glu130. The presence of iron ions in the three-fold pores of RcMf was already established in our previous structure of the trigonal form of RcMf (Bertini *et al.*, 2012), but in that case, the chemical composition of the Fe-complex could not be defined. This represents the first structural evidence of the transit of Fe^{2+}-aqua ions through the ferritin 3-fold pores. The possibility to observe these Fe^{2+} ions only after long times of exposure to iron is probably a consequence of the saturation of the ferritin sites. Indeed, the progressive occupation of the weak binding sites along the path towards the active site may block the incoming Fe^{2+}-aquaions at the pore entrance, in correspondence of the displaced Mg^{2+}-aquaions positions observed in the time 0 structures (iron-free) of both wt-RcMf and RcMf-H54Q variant (see Supplementary).

The 4-fold channels, defined by the four symmetry-related His169, of iron-free structures do not contain metal ions but only chloride from the crystallization solution as indicated by the anomalous signal present there (see Figure 5A). After 2 minutes exposure to the iron salt, the anomalous maps of both wt-RcMf and H54Q variant, demonstrate that one iron ion is bound to the four His169 that define the entrance of the 4-fold channel (see below and Figure 5B). The occurrence of iron binding is also indicated by the conformational change experienced by the side chains of His169, which move at coordination distance (2.43 Å) from the metal ion. This Fe^{2+} ion displays elongated tetragonal coordination geometry with one chloride anion at one of the apices, one water molecule/hydroxide at the other, facing the ferritin cavity, and the four His169 bound in the basal plane (see Figure 5B). The presence of the chloride anion is demonstrated by the signal present in the anomalous difference map obtained from data collected at energy below the Fe K-edge.

Our structural findings of the Fe^{2+}-aqua ion penetrating the ferritin 3-fold channels, together with the $[Fe(His)_4Cl(H_2O)]^+$ (or $[Fe(His)_4Cl(OH)]$) adduct, located at the 4-fold axes, correlate well with the observed loss of oxidoreductase activity upon mutation of Asp127 and Glu130 to hydrophobic residues (Haldar *et al.*, 2011; Theil *et al.*, 2014; Tosha *et al.*, 2012; Yang *et al.*, 2000), and with unaltered catalytic activities of the H169F that, in contrast, displays a slightly inhibited mineral dissolution (Theil *et al.*, 2014). Our data provide the structural rationale of the above findings indicating that the 3-fold pores are routes for Fe^{2+}-aquaion entrance and that the Fe^{2+} substrate cannot penetrate the cage cavity from the 4-fold channels. Conversely, the Fe^{2+}coordination at the 4-fold pores provides a possible model for the interaction that leads to the release of Fe^{2+} from the ferritin mineral. Iron release from the biomineral *ex-vivo* requires iron reduction to Fe^{2+}; this cannot occur under our experimental conditions. Therefore, the Fe^{2+} at the 4-fold pores should come from the Mohr's salt. When it reaches the pore, the chemical characteristics of the locus (hydrophobicity, presence of chloride anion) determine the formation of the observed adduct. In other words, the Fe^{2+} ions stops at the entrance of the 4-fold pore and cannot proceed further, contrarily to what happens at the 3-fold pores.

4. Discussion.

By allowing free diffusion of Fe^{2+} ions in ferritin crystals for different times, followed by flash-freezing, we have been able to obtain a series of time-dependent crystal structures that permit to follow the progressive uptake of Fe^{2+} ions by RcMf and many of the steps along its pathway to reach the OS.

The central portion of each subunit, which hosts the OS, is rich in amino acids that can act as metal ion ligands; each of them can assume multiple conformations, which change depending on the presence of metal ions (Figure 1, all times). In the present time-dependent study we have established the structure of the Fe1 and Fe2 sites with the substrate Fe^{2+} bound. In the time-dependent experiments conducted at pH 8.0, where we consistently measured Fe1-Fe2 distances ranging between 3.5-3.8 Å that are the same, within experimental error, as those observed in the Fe-RcMf crystal grown and maintained in the absence of the second substrate O_2, strongly supporting the presence of diferrous clusters at the oxidoreductase site in all instances (Table S2). At pH 6.5, instead, we repeatedly observed (Bertini et al. 2012, and this work) shorter Fe-Fe distances, characteristic of ferric-μ-oxo/μ-hydroxo clusters. These observations are consistent with the pH dependence of Fe^{2+} oxidation kinetics by RcMf and variants reported by Liu & Theil (Liu & Theil, 2004) that showed an increase of about 100 times in the rate of formation of the diferric product and of the DFP intermediate in going from pH 6.0 to 8.0. The slower release of the diferric product from the RcMf oxidoreductase site in solutions near neutrality allows its direct observation in the crystal (confirming Mössbauer data on diferrous precursors (Krebs *et al.*, 2002b; Krebs *et al.*, 2002a)). In other words at neutral pH, *in chrystallo*, the rate limiting step appears to be the formation and release of the diferric product from the oxidoreductase sites and therefore in crystals at pH 6.5 we can observe species with short intermetal distance characteristic of diferric species. On the other hand, the higher turnover occurring at high pH makes binding of iron at Fe1 and Fe2 sites the rate limiting step of the reaction.

The partial occupancy of all Fe^{2+} binding sites and the time dependence of this structural parameter indicate their transient character and intrinsically low thermodynamic stability in agreement with the measurements performed by ITC on HuHf and *P. furiosus* ferritin (Bou-Abdallah *et al.*, 2002; Bou-Abdallah *et al.*, 2003; Honarmand *et al.*, 2012). All together these data define the path of Fe^{2+} ions towards the reaction site, with iron entering inside the bundle and reaching the oxidoreductase site shuttled by wt-RcMf His54 (Gln54 in RcMf-H54Q and, most probably, Gln58 in HuHf) and then moving to reach the binuclear reaction center constituted by Fe1 and Fe2 sites.

Fe1, with its characteristic square pyramidal geometry, is the first site to become populated. This finding identifies Fe1 as the site with the highest thermodynamic affinity for Fe^{2+}. Once Fe1 is significantly populated, incoming Fe^{2+} binds to the second strongest site (Fe2). When both Fe1 and Fe2 are populated, we start observing Fe^{2+} at the other lower affinity sites. Fe^{2+}, which is continuously diffusing towards the reaction site, accumulates at different positions along its path trapped at sites defined by side chains that can act as transient ligands assisting iron trafficking

inside the protein. At times longer than 15 minutes the occupancy of sites Fe1 and Fe2 decreases, suggesting depopulation of these two sites due to reaction turnover. As soon as the diferricoxo/hydroxo product is formed, the diferric cluster leaves the oxidoreductase site, which is then ready for the next catalytic cycle.

Our data provide the structural evidence that the natural substrate Fe^{2+} species that transits through the entry channels at the 3-fold pores is $[Fe(H_2O)_6]^{2+}$, where they are observed to interact with the side chains of Asp127 and Glu130. These residues have been proposed to exert an electrostatic attraction on the incoming Fe^{2+} species. A large body of evidence accumulated over the years with a variety of experimental techniques on different eukaryotic ferritins indicates the key role played by these residues in allowing the Fe^{2+} ions to enter into the ferritin cage and reach the OS as reported in Table S1 and references therein. In summary, the substitution of these carboxylate residues with hydrophobic ones (Ala or Ile) sensibly reduces the enzymatic activity and the effect has been interpreted in terms of reduced uptake of Fe^{2+} by the nanocage in their absence. The potential energy profiles of divalent cations in the 3-fold channels of ferritin have been calculated (Takahashi & Kuyucak, 2003), providing the theoretical support to the results obtained on mutants. From the bottom of the 3-fold pores, the ferrous aqua ions then proceed inside the inner cage cavity via electrostatic attractions exerted by Glu136-Asp140 (Behera & Theil, 2014) and the oxidoreductase site residues as summarized in Figure 6. The present results also support the role of Glu136 and Asp140 in controlling the access of iron to the catalytic site.

Finally, a Fe binding site is identified at the 4-fold symmetry pores, provided by the four symmetry-related His169. The metal binding residue His169 is conserved in all catalytically active vertebrate ferritins (H or M subunits). The 4-fold pores (and His169 in particular) do not play any role in iron uptake but are possibly involved in iron release (Theil, 2011; Theil *et al.*, 2014). Therefore, the observed Fe at the 4-fold pores represents a model of off-path ions with respect to the catalytic process or of the release of iron from the stored iron mineral core, which occurs under the effect of reductants.

The structural characterization of Fe^{2+} trapped at multiple binding sites along its pathway toward the catalytic center and of the oxidoreductase reaction intermediates allows us to revisit the available structures of ferritin loaded with metal ions different from the natural metal substrate and those of iron-loaded ferritins from different organisms (Ebrahimi *et al.*, 2012; Marchetti *et al.*, 2009). We identify the presence of multiple (Fe3-Fe5) binding sites in the close proximity of the catalytic center, and interpreted them as snapshots of iron moving along its path towards the reaction sites. The analysis of the conformation of side chains acting as iron ligands indicates them as mutually exclusive centers. This finding clearly conflicts with the assumption of previous proposals of the existence of only three iron binding sites in the OS in eukaryotic, prokaryotic and archaeal ferritins (Honarmand *et al.*, 2012). Our structures tell that site 1 has always higher occupancy by Fe^{2+} and that it corresponds to the high affinity site identified in other studies (Ha *et al.*, 1999; Hwang, 2000). On the other hand the almost full occupancy of site1 by Fe^{2+} is reached only in anaerobic crystals, while in presence of dioxygen it is always significantly lower, suggesting

that this site participates to turnover. Our observation that Fe^{2+} occupancy of site 2 is always lower than site 1 provides indication that as soon as site 2 is occupied Fe^{2+}can be oxidized in both sites 1 and 2 leaving a fraction of Fe^{2+} ions bound to site 1. Our data do not definitely exclude that Fe^{2+} oxidation can independently occur in other sites, like, for example, sites 3 and 4; but clearly indicate the presence of multiple alternative iron binding sites rather than a unique "third" site postulated by others (Ebrahimi *et al.*, 2013; Honarmand *et al.*, 2012). The variability of these additional sites in different proteins (and in their functional mutants like H54Q) does not support the proposal that they can be part of an universal mechanism (Honarmand *et al.*, 2012), but is rather in favor of their role as protein-specific modulators of the oxidoreductase reaction kinetics (Bou-Abdallah *et al.*, 2014).

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Figure 1 Effect of different exposure times to Mohr's salt on metal ion binding to wt-RcMf crystals.

wt-RcMf crystallized with $MgCl_2$ (time 0 structure) and iron binding at 1, 2, 15, and 30 minutes exposure. Magnesium and iron ions are represented by light and dark green spheres respectively (arbitrary radius). The anomalous difference map contoured at 5.0 σ, shown as copper wire, is superimposed on the iron ions. To highlight the speciation capability of the measurements, panel Time 2shows the side chains of Met19 and Met33 with superimposed the two anomalous maps obtained at the Fe K-edge (copper wire) and below the Fe K-edge (white wire). The Fourier maps corresponding to other atoms have been omitted for clarity. The amino acids involved in iron binding are shown as sticks. Double conformations of the side chains of residues involved in metal ion binding are also shown. Water molecules are shown as small red spheres. Mg^{2+} coordination bonds are shown as dashed lines. Fe^{2+} coordination bonds are shown as continuous lines. The last panel reports a scheme summarizing the positions of the five Fe^{2+} binding sites observed in average RcMf (and H54Q variant). Protein ligands are represented by sticks of different colors. Fe ions are represented as spheres color coded on their protein ligands. Ligands: Fe site 1 (coral): Glu23, Glu58 (bridging ligand) and His61; Fe site 2 (magenta): Glu58 (bridging ligand) and Glu103; Fe site 3 (green) involves His54 in RcMf or Gln54 in RcMf-H54Q and also Glu57 and Glu103 (bonds not shown for clarity). Fe site 4 (blue) is located on the subunit surface facing the ferritin cavity and involves binding to Glu57, Glu136 and Asp140. The position of His54 is structurally coincident with Gln58 of human H ferritin shown as lilac sticks. Fe site 5 (yellow), found in the ferritin variant H54Q (see Fig. 3, Time 1): Glu53 and Glu57 assume alternative conformations from wt-RcMf.

Figure 2 Oxidoreductase site in wt-RcMf crystallized without the second substrate dioxygen, after 3 minutes exposure to the iron substrate (Mohr's salt).

Four Fe^{2+} ions (represented by green spheres) are clearly seen bound to the enzyme site. The anomalous difference map, obtained from data collected at the Fe K-edge, is superimposed (copper wire). The four sites are coincident with those found after 15 minutes of iron free diffusion under aerobic conditions (Figure 1 time 15).

Figure 3 Effect of different exposure times to Mohr's salt on metal ion binding to RcMf-H54Q variant.

The four panels show the Mg-bound RcMf-H54Q variant (time 0) and the Fe binding sites populated at different times of 1, 15, 60 minutes of iron treatment. Fe^{2+} ions are represented by green spheres of arbitrary radius with the anomalous difference map superimposed contoured at 5.0 σ, shown as copper wire. The Fourier maps corresponding to other atoms have been omitted for clarity. The amino acids involved in iron binding are shown as sticks. Water molecules are shown as small red spheres. The additional Fe5 site is shown in panel Time 1.

The last panel reports a scheme resuming the positions of the five Fe^{2+} binding sites observed in average RcMf (and H54Q variant). Protein ligands are represented by sticks of different colors. Fe^{2+} ions are represented as spheres color coded on their protein ligands. Ligands: Fe site 1 (coral): Glu23, Glu58 (bridging ligand) and His61; Fe site 2 (magenta): Glu58 (bridging ligand) and Glu103; Fe site 3 (green) involves His54 in RcMf or Gln54 in RcMf-H54Q and also Glu57 and Glu103 (bonds not shown for clarity). Fe site 4 (blue) is located on the subunit surface facing the ferritin cavity and involves binding to Glu57, Glu136 and Asp140. The position of His54 is structurally coincident with Gln58 of human H ferritin shown as lilac sticks. Fe site 5 (yellow), found in the ferritin variant H54Q: Glu53 and Glu57 assume alternative conformations from wt-RcMf.

Figure 4 Views along (A) and perpendicular to (B) the 3-fold pore of the 60 minutes RcMf-H54Q structure (the same arrangement is seen in the analogous wt-RcMf structure).

The two Fe^{2+} hexa-aqua ions are sitting on the 3-fold axis. The iron ion are represented as green spheres superimposed to the anomalous difference map obtained from data collected at the Fe K-edge (copper wire) and to the 2Fo-Fc Fourier difference map contoured at 1.2 σ (blue wire). The coordinating water molecules are represented as red small spheres. Superimposed are also H-bonds involving the nearby residues, shown as dashed lines. The open triangles and the continuous line show the position of the crystallographic 3-fold axis.

Figure 5 Views of the 4-fold pores in wt-RcMf (the same occurs in the H54Q RcMf variant).

(A) The 4-fold pore in the iron-free structure. Only a chloride anion is present in the pore at contact distance with the four symmetry related His169 residues. (B) The four-fold pore as it appears after 2 minutes of treatment with the Mohr's salt and onwards. The species $[Fe(His)_4Cl(H_2O)]^+$or$[Fe(His)_4Cl(OH)]$ is present in the pore as clearly shown by the electron density (2Fo-Fc difference Fourier map contoured at 1.2 σ as blue wire). The chemical composition of the complex is revealed by the two anomalous difference maps superimposed (the copper wire refers to the anomalous difference map obtained at the Fe K-edge. The magenta wire refers to the anomalous difference map obtained below the Fe K-edge).

Figure 6 The travel of Fe^{2+} ions inside the RcMf subunit.

The about 50 Å long probable pathway of Fe^{2+} ions, from the 3-fold axis channel to the oxidoreductase site as indicated by the crystallographic structures. The residues involved in Fe^{2+} binding (green spheres) are shown as sticks. The two Fe^{2+} ions bound to Fe1 and Fe2 sites are shown as red spheres. The Fe^{2+} trapped at the 4-fold axis pore is shown as a green sphere.