Chemistry at the protein-mineral interface in L-ferritin assists the assembly of a functional (μ3-oxo)Tris[(μ2-peroxo)] triiron(III) cluster

Chemistry at the protein-mineral interface in L-ferritin: assisted assembly of a functional (μ^3-oxo)tris[(μ^2-peroxo)] triiron cluster

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Abstract

X-ray structures of homopolymeric L-ferritin obtained by freezing protein crystals at increasing exposure times to a ferrous solution showed the progressive formation of a triiron cluster on the inner cage surface of each subunit. After 60 minutes exposure, a fully assembled (μ^3-oxo)tris[(μ^2-peroxo)(μ^2-glutamato-κ*O*:κ*O*')](glutamato-κ*O*)(diaquo)triiron(III) anionic cluster appears in human L-ferritin. Glu60, Glu61 and Glu64 provide the anchoring of the cluster to the protein cage. Glu57 shuttles towards the cluster incoming iron ions. We observed a similar metallocluster in horse spleen L-ferritin, indicating that it represents a common feature of mammalian L-ferritins. The structures suggest a mechanism for iron mineral formation at the protein

interface. The functional significance of the observed patch of carboxylate side chains and resulting metallocluster for biomineralization emerges from the lower iron oxidation rate measured in the E60AE61AE64A variant of human L-ferritin, leading to the proposal that the observed metallocluster corresponds to the suggested but yet unobserved nucleation site of L-ferritin.

Significance Statement: Iron is an essential element in biology but has limited bioavailability. Ferritins are 24-mer iron-storage nanocage proteins that concentrate iron in their inner compartment as a bioavailable iron oxide biomineral. In L-type subunits, abundant in ferritins from organs involved in long-term iron storage, the biomineralization has been proposed to proceed through nucleation events involving iron(II) oxidation at the inner cage surface. Here we demonstrate the nature and structural features of these nucleation sites. Structures captured during iron uptake show that the formation of the iron biomineral proceeds via the assembly of a tri-nuclear iron cluster, anchored to the protein via glutamic acid side chains, and involving oxo and peroxo ligands that are produced during the iron(II) oxidation by dioxygen.

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Twentyfour-mer ferritins are ubiquitous iron-biomineralizing nanocage proteins. In mammals, they are generally heteropolymers composed by two different types of subunits, the heavy H and the light L (183 and 175 amino acids, respectively, in the human chains). The subunits self-assemble to form a hollow structure with a central cavity capable of accommodating thousands of iron atoms in the form of an oxoferric biomineral (1, 2) (Fig. 1*A*). The relative ratios of the two types of subunits in the 24-mers vary in different tissues; ferritins in iron storage organs, such as liver and spleen, are richer in L-subunits, while those with fast iron metabolism such as brain and heart are richer in H-subunits (3). The H-subunit contains a ferroxidase center, composed by the so-called Fe1 and Fe2 sites, capable of rapidly oxidizing Fe^{2+} to Fe^{3+} (4, 5); a relatively low number of amino acid ligands characterizes the two sites. In human H-subunit, Glu62 acts as a bridging ligand between the two metals; the metal at Fe1 is also bound to His65 and monodentate Glu27 while the metal at Fe2 binds bidentate Glu107 (4). Accessories transient metal sites (Fe3, Fe4 and, in some cases, Fe5) have been identified by X-ray crystallography in the proximity of the ferroxidase site and they have been demonstrated to play a key role in the reaction turnover (4, 6). L-subunits lack the ferroxidase site, whereas a number of other residues are conserved with respect to the H-subunit (homology 53% between human L- and H-chains; see Fig. 1*B*) (7). Consequently, iron incorporation in nanocages rich in L-subunits is much slower. Nevertheless, even homopolymeric L-ferritins are able to biomineralize iron. The proposed mechanism involves the presence of a

putative nucleation site on the inner cage surface of L-subunits. In several studies, the role of residues Glu57, Glu60, Glu61, and Glu64 (human H-chain aligned numbering, Fig. 1*B*), for an efficient biomineralization was inferred by site directed mutagenesis or chemical modifications (8, 9). Non-physiological metal ions like Cd^{2+} have been observed bound to the corresponding glutamates of the above mentioned human Glu residues in several mammalian L-ferritins (10-14), while the structure with iron of these homopolymeric L-ferritins has not been yet reported.

Here, we describe a structural study of iron binding to human and horse L-ferritins (HuLf and HoLf, respectively), were we observe the ferritin-mediated assembling of a tri-nuclear iron cluster on the inner surface of L-ferritin cages with a scaffold provided by Glu60, Glu61 and Glu64 ligands. The presence of these residues facilitates the biomineralization process, as demonstrated by different kinetics of biomineral formation between recombinant wild type (WT) homopolymeric HuLf and its triple variant E60AE61AE64A. All together, these results provide a direct evidence of the glutamate-bound triiron cluster as the mineral nucleation site in L-ferritins. The cluster structure is unprecedent in biological systems although it shows striking structural similarities to a synthetic hexanuclear iron cluster proposed about 20 years ago as a possible model of ferritin biomineral (15).

Results

Structural Studies on Iron-free HuLf and HoLf. Overall Structures. Crystals of iron-free HuLf were exposed to a concentrated ferrous ammonium sulfate (Mohr's salt) solution and frozen after 15, 30 and 60 minutes of diffusion of Fe^{2+} ions into the crystals. The same experimental approach was used to characterize the iron-loaded state of the HoLf ferritin. The structures of HuLf and HoLf before iron exposure were determined as reference structures. They show the typical quaternary structure of maxi-ferritins, consisting of the 24-subunit assembly to form a hollow cage. The asymmetric unit in both structures contains a single protein chain, representing the spatial and time average of all the molecular subunits found in the crystals. Each ferritin subunit shows the characteristic four-helix bundle tertiary structure completed by a fifth helix (Fig. 1*A*) (13, 16). The protein models are almost complete with the exception of the two initial and the three final residues of HoLf and the two starting residues in the HuLf sequence. The structure of the purified horse spleen ferritin is that of an L-chain homopolymer.

Several cadmium ions have been observed bound to both iron-free HuLf and HoLf, in the 3-fold axis channel, on the 2-fold axis and on the internal surface of the protein shell, but none on the 4-fold axis channel (see SI Channels and Metal Ions Binding Sites) (17, 18). In the iron-free structures of both L-type ferritins two Cd^{2+} ions are coordinated by Glu57 and Glu60, and by Glu61 and Glu64, respectively.

L-ferritin Subunits Provide the Scaffold for a Trinuclear Peroxo-bridged Cluster. Despite several structures of HuLf and HoLf ferritins have been determined in complex with different metal ions (13, 16, 19), no structural observation of iron binding to the mineralization site have been reported in the literature, the only iron(III) ion reported in HoLf being bound to an exogenous ligand (20). To the goal of obtaining insights on the ferritin biomineralization process, we have obtained the crystal structures of the iron-loaded HuLf and HoLf cages. Besides observing the expected iron ions in the access 3-fold channels (SI Channels and Metal Ions Binding Sites), we could monitor iron binding inside the cavity. Iron binding to the inner cage surface is achieved with the involvement of the carboxylic side chains of three glutamic acids, namely Glu60, Glu61, and Glu64 in HuLf and the corresponding residues in HoLf (Fig. 2 and 3). Three iron ions are coordinated to the above carboxylate side chains, generating an oxo-centered trinuclear cluster (Fig. 2). The anomalous difference maps show that the triiron cluster is formed at 30 minutes of iron exposure (the 15 minutes structure does not show any iron atom bound), but the best defined ligand set of the iron ions appears in the structures obtained after 60 minutes (Fig. 3*A*-*D*), which will be described in detail below. Fourier difference maps of both HuLf and HoLf show the presence of an atom in the middle of the iron cluster that has been interpreted as an oxide anion forming a μ^3-oxo bridge (Fig. 3*B*,*D*; see SI Structural Features of the Metal Cluster). In the 60 minutes structure of HuLf (1.98 Å resolution) the oxide anion is located almost centrally in the plane formed by the three iron ions with coordination distances in the 1.8 – 2.0 Å range, all identical at 2σ significance level (Fig. 2 and 3*B,* Table S3). The coordination displayed by the iron ions in the cluster is a distorted octahedral geometry. The carboxylate groups of Glu60, Glu61 and Glu64 symmetrically bridge the three Fe couples (Fig. 2) on the same side of the cluster. On the opposite side, each pair of iron ions results connected by an elongated electron density compatible with a diatomic molecule. The observed densities have been interpreted as due to three peroxide anions generating a second series of bridges (Fig. 3*B*). The arrangement of the three carboxylates, the three iron ions and the three peroxides follows an almost regular 3-fold symmetry centered on the oxide anion. A sixth ligand is present in the basal plane of each Fe ion: a water molecule for Fe1 and Fe2, and an oxygen from the carboxylate group of Glu57 (monodentate) for Fe3. Glu57 bridges a nearby fourth iron aqua-ion present at lower occupancy (about 50 %). A likely explanation of the described arrangement of the iron ions in this site, is that we have detected a (μ^3-oxo)tris[(μ^2-peroxo)(μ^2-glutamato-κ*O*:κ*O*')](glutamato-κ*O*)(diaquo)triiron(III) anion. Remarkably, the L-ferritin cluster shows the same structure of the analogous portion present in a synthetic hexanuclear iron(III) cluster reported as a model of the ferritin mineral core (a detailed structural comparison is reported in SI Structural Features of the Metal Cluster and Table S3) (15). The striking similarity to that model compound supports the presence of iron ions in the ferric state resulting in an overall trinegative charge of the assembly in HuLf, which can provide the driving force to attract new iron(II)

ions to promote oxidation and biomineralization, as exemplified by the observed fourth iron ion approaching the cluster via Glu57.

The similarity between the trinuclear iron cluster in L-ferritin and the above model extends also to spectroscopic properties. The UV-vis spectrum of the model compound is characterized by the absence of any spectral feature between 500 and 800 nm and the presence of poorly defined shoulders (at 408, 456, and 534 nm) superimposed on a broad underlying tail (15). In HuLf a broad shoulder at 490 nm is present that forms in the crystals after soaking times corresponding to those of the formation of the trinuclear cluster observed by us in crystal structures of HuLf and HoLf (see new Fig. S3). Such feature is absent in the spectra of crystal of HuHf exposed to Fe^{2+} for the same time.

The comparison with the iron-free HuLf structure reveals that the formation of the oxo-centered trinuclear iron cluster occurs without modification of the protein fold, but with a rearrangement of the glutamate side chains involved in iron coordination. The Cd^{2+} ions present in the same site in the iron-free structure are displaced by iron. Upon formation of the iron cluster the carboxylate of Glu57 shifts by ca 2.5 Å (and rotates on the β-carbon by 60°) whereas the carboxylate of Glu60 moves by about 1.3 Å (and rotates on the γ-carbon by 50°). The formation of the cluster induces also the rearrangement of the side chains of several surrounding residues. In particular, the hydrophilic side chains of Arg68 and Glu140 contribute to stabilize the cluster through the formation of a network of water-mediated interactions involving Glu61 and the water molecule indicated as WatL in Fig. S2. The structures of HoLf exposed to an iron salt solution for the same time intervals confirm the formation of the oxo-centered trinuclear iron cluster in the mineralization site of L-type ferritins (Fig. 3*C*,*D* and Table S3). Only two peroxide anions can be modelled bridging the Fe1-Fe2 and Fe2-Fe3 couples (Fig. 3*D*). No density is present in the omit map in correspondence to the third peroxide observed in HuLf. Only an elongated maximum is found close to Fe1 and incoming Fe4 that has been interpreted as a water molecule. The lower resolution of this structure (2.22 Å) does not provide a satisfying modelling of all aqua-ligands that should complete the iron coordination in the cluster. However, the arrangement of the three iron ions in the cluster and of the incoming fourth iron ion, at lower occupancy, is almost superimposable with that present in HuLf (Fig. 3*A*,*C*). This confirms that the formation of such cluster is a general feature of L-subunits and suggests its functional relevance.

Assessing the Functional Relevance of the Trinuclear Iron Cluster. The crystal structure indicates the key role of Glu60, Glu61 and Glu64 to act as template for the formation of the cluster. Our functional working hypothesis was that substituting these negatively charged glutamate residues with non-polar alanine could alter the biomineralization process. To prove the functional role of the residues linking the triiron cluster to the protein matrix, we designed and produced the triple mutant E60AE61AE64A. The protein yield was 30 mg/L, meaning that these multiple mutations did not affect the expression efficiency. Moreover, the typical

quaternary structure was maintained as demonstrated in size exclusion chromatography experiments (Fig. S1). A comparative kinetic analysis of the formation of ferric biomineral between WT HuLf and its variant E60AE61AE64A was performed spectrophotometrically. Temporal spectral changes monitored A_{350nm} (see Materials and Methods) after the addition to protein solutions of different amounts of Fe^{2+} ions (3-18 Fe^{2+} per subunit) in 200 mM MOPS, 200 mM NaCl pH 7 were acquired in parallel (Fig. 4). The same measurements were repeated also in absence of the protein to observe the contribution of spontaneous ferrous ions oxidation. In the absence of ferritin, precipitation of iron-oxo species is clearly observed through a decrease of the A_{350nm}; the effect is stronger at higher concentrations of Fe^{2+} (Fig. 4*C*,*D*, blue curves). Differently, in the presence of HuLf, either WT or E60AE61AE64A variant, the iron is maintained in a soluble form and the A_{350nm} reaches a plateau at incubation times that are dependent upon the number of added Fe^{2+} equivalents (Fig. 4: red and green curves). The initial velocity of Fe^{2+} oxidation is more rapid in the presence of WT HuLF with respect to the E60AE61AE64A variant, thus demonstrating a direct role of the mutated residues in the iron oxidation kinetics and therefore attributing a functional significance to the trinuclear iron cluster that forms at their site. From the expansion of the initial reaction rate (central panels in Fig. 4*A*-*D*) the differences between the WT and the E60AE61AE64A variant cages are clearly visible, accounting for reductions of the initial reaction rates by 33%, 55%, 68% and 70% at 3, 6, 9, 18 Fe per subunit ratios, respectively. The behavior of the E60AE61AE64A variant is always closer to that of the iron oxidation in the absence of ferritin rather than to that of the WT L-ferritin.

Discussion

It is generally accepted that in ferritin cages lacking ferroxidase-active subunits, the core biomineral formation proceeds via few key steps. The iron entry into the cage cavity proceeds through the iron channels at the 3-fold symmetry axes (Fig. 5) and is followed by oxidation at specific, yet unobserved, nucleation sites that facilitate the subsequent growth of the mineral (2, 4, 21, 22). Using X-ray crystallography, we detected for the first time iron bound at inner cage sites of L-subunits of recombinant homopolymeric human ferritin in the form of (μ^3-oxo)tris[(μ^2-peroxo)(μ^2-glutamato-κ*O*:κ*O*')](glutamato-κ*O*)(diaquo)triiron(III) anion; a very similar arrangement was here observed in HoLf. The protein ligands to iron are provided by Glu60, Glu61 and Glu64, while Glu57 appears to be involved in shuttling additional iron ions towards the cluster. The peroxo cluster is fully formed in HuLf after 60 minutes of Fe^{2+} diffusion through the crystals, while in the lower resolution structure of HoLf at the same diffusion time only two fully formed peroxide bridges could be modelled. The functional role to the observed trinuclear iron cluster, emerges from the comparative kinetic analysis of the ferritin-mediated iron oxidation reaction of WT and E60AE61AE64A variant HuLf cages. The three mutated residues have a clear role in accelerating the oxidation reaction at low iron contents. These results allow us to propose the three iron-carboxylate cluster as the site for the iron mineral nucleation site in L-ferritins. Indeed, the observed

metallocluster appears to be a general characteristic of animal L-type ferritins, as it is found also in HoLf crystals. The chemical features of the observed triiron cluster partially confirm previous proposals about the nature of biomineral precursors. The observed L-ferritin trinuclear cluster can be considered as a portion of the hexanuclear synthetic cluster discussed in the Results section, which was proposed to represent a ferritin biomineral model (15). Other authors have suggested that an oxo-centered trinuclear iron cluster, which forms spontaneously on the surface of class Ib ribonucleotide reductase R2 protein from *Corynebacterium ammoniagenes* in crystals subjected to iron soaking, could mimic an early stage in the mineralization of iron in ferritins (23). In this case, however, the bridging ligands were provided by six carboxylates whereas no peroxide anions were detected (23).

In analogy with the ribonucleotide reductase case, in L-ferritins, the presence of three adjacent Glu residues at positions 60, 61 and 64 is perfectly suited to provide cooperative binding of iron in a trinuclear center. In H-type subunits, like human H-ferritin (HuHf), this ideal arrangement of carboxylate side chains is disrupted by replacement of Glu60 with a His, with the concomitant increase in the conformational freedom of Glu61 side chain (6). Also Glu57 is replaced by His in HuHf (Fig. 1*B*). Consistently, the biomineralization reaction in H-ferritin cages does not require a nucleation site (24, 25) and His57 and Glu61 are involved in controlling the access of ferrous ions to the ferroxidase site (6); the reaction at the ferroxidase site is much faster than in L-subunits, with time scales for the iron oxidation < 1 s. Even a low amount of H-subunits (1-2 per cage) the reaction rate is dominated by the catalytic oxidation of iron occurring at the ferroxidase sites. The formation of ferric clusters of high nuclearity in ferroxidase active (H or H') subunits has been inferred from Mössbauer (26-28) and NMR data (29, 30), but they have never been detected by time-lapse anomalous crystallography approaches. This supports the occurrence of a different iron mineralization process in ferroxidase active subunits leading to spatially disordered clusters that escape detection by X-ray crystallography. The observed peroxide ligands in the L-ferritin cluster might result from the initial oxidation of ferrous iron bound to the nucleation site and by the excess reductant (Fe^{2+}) present in solution (31). The formation of a peroxide-complex in L-ferritins supports a mechanism for the mineral growth in the absence of the ferroxidase catalytic site, where the reaction between glutamate-bound Fe^{2+} and dioxygen provides the Fe-peroxide anionic catalyst able to attract and oxidize incoming Fe^{2+} aqua-ions. This mechanism is consistent with the previous proposal (31) that, at iron load up to 500 Fe per cage, hydrogen peroxide is involved in iron oxidation while, at higher Fe/protein ratios, the core biomineralization occurs via iron deposition directly on the biomineral surface (crystal growth model). The peroxide-driven reaction could be relevant in the contest of the proposed ferritin-mediated detoxification reaction involving both Fe(II) and H_2O_2 (31). *In vivo,* this reaction is important to attenuate harmful Fenton chemistry under conditions of iron overload. *In vitro*, it becomes the dominant one

due to the lack of the protective role exerted by catalase that could disproportionate any H_2O_2 produced upon iron oxidation. Time-lapse crystallography shows here once more (4, 6, 32) its merit in capturing reaction intermediates within ferritin cages, thus leading to the identification of functional iron-binding sites and to a dynamic picture of their population by iron ions.

Materials and Methods

Protein Preparation. The plasmid encoding WT HuLf (33) was transformed into *E. coli* BL21(DE3)-pLysS competent cells. The protein was expressed and purified following a home-made protocol developed for HuHf (34) with some modifications (17°C with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 48 hours in rich medium Luria Bertani, LB). The subunit mass was analyzed by MALDI mass spectrometry. The retention volume obtained in the size exclusion chromatography demonstrated the 24-mer formation (Fig. S1). The E60AE61AE64A HuLf variant was expressed and purified as for WT; mutations were verified by MALDI peptide fingerprint analysis.

HoLf samples were obtained by purification of the commercially available protein.

Details on proteins' preparation, purification, initial characterization and mineralization are provided in the *SI Materials and Methods*.

UV-Vis measurements. The rate of the biomineralization reaction in solution was determined spectrophotometrically upon addition of variable amounts of Fe^{2+} (3-18 Fe^{2+} per subunit). The characteristic absorbance at 490 nm was measured in HuLf crystals. Experimental details are provided in *SI Materials and Methods*.

Crystallography. A series of L-ferritin structures were determined by soaking HuLF and HoLf ferritin crystals with a freshly prepared $[(NH_4)_2Fe(SO_4)_2]\cdot 6H_2O$ solution for exposure times of 15-60, minutes, followed by flash freezing in liquid nitrogen. The procedure, detailed in the *SI Materials and Methods*, follows with some modifications an established protocol that already allowed us to characterize iron oxidation in the ferroxidase site of human H- and *Rana Catesbeiana* H'- type ferritins (4, 6). Data collection, structure solution and refinement procedures are given in the *SI Materials and Methods* and in Table S1 and S2. The final coordinates and structure factors for 60 minutes iron-loaded HuLf and HoLf have been deposited in the Protein Data Bank under the accession codes 5LG8 and 5LG2, respectively.

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Figure legends

Fig. 1. Ferritin main structural features and sequence alignment. (*A*) *Left*, cartoon representation of the four-helix bundle subunit: helices III and I are solvent-exposed and linked by a long loop (*magenta*), helices II and IV locate on the inner cage surface; a fifth short helix at the C-terminal is tilted of 60° with respect to the bundle axis. *Right*, section of the human L-ferritin (HuLf) cage. The trinuclear iron clusters sprout into the 8 nm cavity, surrounded by the protein shell of 12 nm external diameter. (*B*) Alignment of amino acid sequences of human H-ferritin (HuHf), HuLf and horse spleen L-ferritin (HoLf) (FRIH_HUMAN, FRIL_HUMAN and FRIL_HORSE, respectively), performed by Clustal Omega. All sequences are depleted of the initiator methionine. In light blue, the residues binding the metal cluster in L-ferritin. In orange, the iron-binding amino acids in the ferroxidase site of the catalytically active H-subunit and the corresponding amino acids in the L-chains of HuLf and HoLf (both lacking the ferroxidase site). In magenta, the amino acids in the conserved pore responsible of iron entry.

Fig. 2. Different stereo-views of the trinuclear iron binding site in HuLf. (*A*) Electrostatic surface representation of the trinuclear cluster binding site. Iron ions and water molecules are represented as cyan and red spheres,

respectively. Peroxide molecules are shown as red ball-and-stick models. (*B*) Structure of the oxo-centered trinuclear iron cluster. Coordinating glutamates are highlighted as sticks. Iron ions, water and peroxide molecules are represented as in "*A*". Coordination bonds to iron ions are shown as dashed red lines; coordination distances (Å) are also displayed. Two different orientations are proposed in panels *A* and *B* to better appreciate the structural features of the protein site and metal cluster.

Fig. 3. Stereo-views of the iron cluster in HuLf and HoLf. (*A*) Iron ions (*cyan spheres*) in HuLf are surrounded by the anomalous difference map (*dark red mesh*) contoured at 4.0 σ. (*B*) Omit map (*green mesh*) in HuLf contoured at 3.0 σ of peroxide and oxide anions involved in iron coordination. In both HuLf pictures: helix II, light blue cartoon; residues involved in iron coordination, light blue sticks. (*C*) Iron ions (*cyan spheres*) in HoLf are surrounded by the anomalous difference map (*dark red mesh*) contoured at 5.0 σ. d, Omit map (*green mesh*) in HoLf contoured at 3.0 σ of peroxide and oxide anions, and the water molecule involved in iron coordination. In both HoLf pictures: helix II, yellow cartoon; residues involved in iron coordination, yellow sticks.

Fig. 4. Kinetic measurements in WT and E60AE61AE64A HuLf variant. Kinetics of oxo-ferric species formation followed spectrophotometrically as the change in absorbance at 350 nm over 16 hours (960'). Different concentrations of Fe^{2+} were incubated, at room temperature, in 200 mM MOPS 200 mM NaCl pH 7 buffer with 25 µM protein subunits of WT HuLf (*red curves*) and triple mutant E60AE61AE64A (*green curves*) or without protein (blank, *blue curves*). Addition of: (*A*) 3 Fe^{2+} ions per subunit; (*B*) 6 Fe^{2+} ions per subunit; (*C*) 9 Fe^{2+} ions per subunit; (*D*) 18 Fe^{2+} ions per subunit. The curves have been recorded over 960 min (left panels), the expansion of the curves during the first 100 min are provided in the central panels, to highlight the differences between WT and triple mutant E60AE61AE64A. The bar plots of the right panels provide the % reduction in ferric-oxo species formation rate in the triple mutant with respect to 100% rate assumed for the WT; rates were calculated from the linear fitting of the first 30 min (3-9 Fe^{2+} per subunit) and 15 min (18 Fe^{2+} per subunit).

Fig. 5. The properties of the 3-fold ion channel in ferritin. Electrostatic surface representation of a trimer of L-ferritin subunits where three monomers (*gray cartoon*) come in contact generating a 3-fold channel responsible for iron entry. The external (*left*) and the internal (*right*) views of the pore circled with black lines

show the negatively charged environment, due to the presence of conserved Asp131, Glu134 and Thr135 side chain residues. In HoLf, in position 135 there is a Ser, which is highly similar to Thr.