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Comparative Fe and Zn K edge X-ray absorption spectroscopic study of the ferroxidase
centres of Human H-chain ferritin and bacterioferritin from Desulfovibrio desulfuricans.

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**Abstract**

Iron uptake by the ubiquitous iron storage protein, ferritin, involves the oxidation of two Fe(II) ions located at the highly conserved dinuclear "ferroxidase centre" in individual subunits. We have measured X-ray absorption spectra (XAS) of four mutants (K86Q, K86Q/E27D, K86Q/E107D, and K86Q/E27D/E107D, involving variations of Glu to Asp on either or both sides of the dinuclear ferroxidase site) of recombinant Human H-chain ferritin (rHuHF) in their complexes with reactive Fe(II) and redox-inactive Zn(II). The results for Fe-rHuHF are compared with recombinant *Desulfovibrio desulfuricans* bacterioferritin (DdBfr) in three states: oxidised, reduced, and oxidised/Chelex®-treated. The X-ray absorption near edge region of the spectrum (XANES) allows the oxidation state of the Fe ions to be assessed. Extended X-ray absorption fine structure (EXAFS) simulations have yielded accurate geometric information that represents an important refinement of the crystal structure of *DdBfr*; most metal-ligand bonds are shortened and there is a decrease in ionic radius going
from the Fe(II) to the Fe(III) state. The Chelex®-treated sample is found to be partly mineralized, giving an indication of the state of Fe in the cycled-oxidised (reduced, then oxidised) form of $Dd$Bfr, where the crystal structure shows the dinuclear site to be only half-occupied. In the case of $rHu$HF the complexes with Zn(II) reveal a surprising similarity between the variants, indicating that the $rHu$HF dinuclear site is rigid. In spite of this, the $rHu$HF complexes with Fe(II) show a variation in reactivity that is reflected in the Fe oxidation states and coordination geometries.

**Introduction**

Ferritins are ubiquitous proteins that concentrate, store, and detoxify intracellular iron. They do this by catalysing the oxidation of Fe(II) at dinuclear ferroxidase centres: the Fe(III) then migrates to nucleation sites within the internal protein cavity, where it is deposited as an inorganic mineral phase. Ferritins are widespread in nature, being found in bacteria and archaea as well as in eukaryotes. Their molecules are mostly assemblies of 24 structurally equivalent subunits related by octahedral 432 symmetry which form a nearly spherical shell of ~ 480 kDa with an 80 Å diameter iron storage cavity in which up to 4500 iron ions can be accumulated. The protein subunits are folded in compact, four-helix bundles [1].

Most vertebrate ferritins are formed from two different subunits, H and L, although amphibians have a third subunit, M. The conserved residues essential for the ferroxidase activity, Glu27, Tyr34, Glu61, Glu62, His65, Glu107, and Gln141 (Human H-chain numbering) are found only in H-type ferritins [2-4]. Crystal structures of the metal centre in recombinant (K86Q, for ease of crystallisation) Human H-type ferritin ($rHu$HF) [5] and in *Escherichia coli* [6] have been reported (see Figure 1A for a schematic representation of the
protein ligands). The di-iron centres in animal H ferritins are relatively labile and the iron could move from the dinuclear site into the iron storage cavity upon oxidation, in line with the aforementioned mechanism of uptake as Fe(II) and storage as Fe(III). In order to avoid this, Zn(II) has been used as a redox-stable alternative to Fe(II) [7] and recently some of us reported crystal structures of the Zn derivatives of rHuHF and some other variants [8].

--- FIGURE 1 ---

Bacterioferritins differ from mammalian-type ferritins in terms of amino acid sequence, immunological cross reactivity, composition of the iron core and particularly by the presence of one haem group per subunit dimer. The bacterioferritin from the anaerobic sulphate/nitrate reducer Desulfovibrio desulfuricans (DdBfr) has in its native form a fully occupied diiron ferroxidase centre (see Figure 1B for a schematic representation), but without a central ferrihydrite core. When isolated from the bacterium under anaerobic conditions it has a total of 60 Fe atoms/ protein molecule, consisting of 24 di-iron centres and 12 haem irons, as shown in the crystal structure [9]. However, although iron in DdBfr is found in the di-iron centre, in principle a larger amount of iron can be stored inside the cavity of the protein.

With its large core full of iron, ferritin was an obvious target for early biological EXAFS studies [10]. More ambitious studies in which apoferritin was reconstituted with just enough iron to fill the dinuclear ferroxidase site, resulting in samples much more dilute in iron, followed later [11]. EXAFS was also used to compare the ferritin core to accurate synthetic models [12]. In a recent stopped-flow EXAFS study on the HuHF ferroxidase site, evidence was presented for intermediates with Fe-Fe distances as short as 2.5 Å in the reaction of the bis-Fe(II) site with oxygen [13]. In order to evaluate the importance of the
distance between the iron ions in the ferroxidase centre in rHuHF for the reactivity, we have
constructed HuHF site directed mutants in which the Glu residues on either side of the
dinuclear iron site, Glu-107 or Glu-27, alone or together, are substituted by Asp. All Human
ferritin proteins studied in this work contained the Lys86 to Gln substitution to allow
crystallisation [5]; for convenience the reference protein rHuHF-K86Q, is referred to as the
“wild type” rHuHF, and the other mutants, in which one or more Glu residues have been
mutated in addition to the K86Q mutation, are named rHuHF-E107D, rHuHF-E27D, and
rHuHF-E107D/E27D. We have recently reported the crystal structures of the recombinant
apoproteins and Zn complexes [8]. In the present study, the Fe in the ferroxidase sites of these
rHuHF mutants and the Zn in their Zn(II) derivatives are compared with the Fe in the
relatively stable dinuclear ferroxidase site in DdBfr by XAS. The DdBfr used in this EXAFS
study is not the wild type DdBfr studied by crystallography [9], but a recombinant produced
in E. coli into which the di-iron (ferroxidase) centre has been reconstituted with Fe and the
haem cofactor is absent. The Zn EXAFS results show that the rHuHF mutants are remarkably
similar in structure, but the Fe EXAFS reveals differences in reactivity. The DdBfr results
show that the Fe-ligand distances are shorter than in the crystallographic study, that the Fe ion
in the reduced state has an ionic radius which is significantly larger than that in the other
states, in line with reduction from Fe(III) to Fe(II), and that the iron in a Fe-containing protein
sample that has been exposed to air is at least partly mineralized.

Materials and methods

Cloning, overexpression and purification of bacterioferritin (DdBfr). For expression
purposes, homologous oligonucleotides that allowed the introduction in the Bfr gene of an
NdeI restriction site, at the start codon, and a BamHI restriction site, downstream of the stop
codon, were designed. By means of a PCR reaction, using the oligonucleotides, Pfu polymerase (Stratagene) and pUBfRd plasmid [14], amplification of the complete Bfr gene (540-bp) was achieved. After purification, the 540-bp fragment of Bfr was cloned in pT7-7, previously cut with the appropriated restriction enzymes, and transformed in E. coli DH5α cells [15]. The resultant recombinant plasmid, pT7Bfr, was isolated and sequenced and ensured the integrity of the gene.

Cells of E. coli BL21-Gold (DE3) (Promega) freshly transformed with pT7Bfr were grown overnight, at 37°C, in Luria-Bertani (LB) medium with ampicillin (100 μg/ml) under aerobic conditions. This overnight culture was used (1%) to inoculate fresh LB media and when the cells reached a cell density of OD600nm=0.8, 400 μM of isopropyl-thio-β-D-galactoside (IPTG) was added. After four hours, cells were harvested by centrifugation and resuspended into 10 mM Tris-HCl, pH 7.5, and broken in a French pressure cell (SLM; Aminco) at 9,000 psi. Analysis of the recombinant cells revealed that the overproduced DdBfr protein was present in inclusion bodies. Hence, inclusion bodies were collected by a low speed centrifugation (5,000 × g for 10 min). The pellet was dissolved in 20 mM Tris-HCl pH 7.6 (buffer A) which contained 8 M urea, 0.5 M NaCl and 5 mM dithiotreitol (DTT) and stirred for 30 min, at room temperature. The insoluble material was then removed by a centrifugation step performed at 17,000 × g during 15 min. For reconstitution purposes, the supernatant that was collected by centrifugation was then dialysed in consecutive steps by successive additions of fresh buffer. To this end, after an initial six hours dialysis in 1 L of urea 8 M, the protein suspension was dialyzed eight times, in time intervals of six hours, in 250 ml of buffer A. A final dialysis in 2 L of buffer A plus 150 mM NaCl was performed during twelve hours. The solution was centrifuged at 5,000 × g, 20 min and the supernatant concentrated in a diaflow apparatus with a membrane YM3 (Amicon). The sample was again centrifuged at 11,600 × g, 30 min, and then applied onto a Superdex column, S-75 (XK26/53),
using buffer A plus 150 mM NaCl with a flow of 0.75 ml/min. After this purification step, the
gene, identified by Western-blotting with antibodies raised against the native Dd
haemoferritin, was found to be pure in SDS-PAGE. The recombinant Dd Bfr was isolated in
apo-form, i.e., with no haem or iron content.

**Bacterioferritin metal loading.** The protein was concentrated and diluted in 0.1 M
MES pH 7.0 with NaCl 0.2 M buffer three times to remove TRIS-HCl from the sample. The
water solution used to dissolve (NH₄)₂Fe(SO₄)₂ was degassed under vacuum for 30 min and
flushed with argon for 30 min. The oxygen free iron stock solution was added to DdBfr to
yield a final concentration of 36.5 mg/ml of 2.1 irons by protein monomer. The iron loaded
sample was left one hour anaerobically before freezing in the sample holder (reduced DdBfr
sample). This reduced iron loaded DdBfr sample was recycled after beam exposure and
allowed to oxidise under air atmosphere for one hour at room temperature. The oxidised
sample was then loaded on the sample holder and frozen for analysis (oxidised DdBfr
sample).

A distinct sample was treated for excess iron removal with a Chelex® resin following
iron loading. The DdBfr was loaded with 2.1 irons per protein monomer in 0.1 M MES buffer
at pH 6.5. It was then left for oxidation for one hour before filtering slowly with Chelex® resin
squeezed into a small syringe. The diluted iron loaded protein gathered was concentrated to
20.5 mg/ml (oxidised/Chelex®-treated DdBfr sample). Iron determination analysis by ICP
(inductively coupled plasma) revealed that the freshly purified recombinant protein was
metal-free and each subunit of the Chelex®-treated sample contained 1.35 iron per protein
subunit.
Human ferritin recombinants. The complete Human ferritin H-chain cDNA was introduced in pAS expression plasmid [16, 17]. The ferritin-encoding DNA region was amplified by PCR containing oligonucleotide primers containing base changes necessary to produce the required mutations: Glu-107-Asp, or Glu-27-Asp, or both. The PCR product was subcloned into the pGEM-T vector using the 3'-T overhangs. The resulting plasmid was electroporated in TG1 and sequenced. Finally the EcoRV-SphI fragment containing the required mutations was reinserted into expression plasmid. Production and purification of recombinant apoferritins are described elsewhere [16].

HuHf metal loading. The metal/protein subunit ratio was fixed to two, in order to saturate the ferroxidase centre and avoid metal complexation in other weaker metal-binding sites. For iron loaded protein, all solutions were degassed 30 min. with argon bubbling except the protein to avoid denaturing. Under argon atmosphere, add 5 µl iron solution (Conc 0.381 M, pH 2, ratio 2:1 per subunit) to 1 ml protein solution (Conc 20 mg/ml, i.e. 3.96 \times 10^{-5} M of protein, 25 mM degassed Mes buffer, pH 6.5). Add 10 % Chelex® to the reaction tube and mix thoroughly, filter the mixture through a 4.5 μm milipore unit. Allow the samples to oxidise under ambient atmosphere for 10 min. For zinc loaded ferritins, add 500 µl zinc solution (ZnCl₂ 2.86 mM) to 25 ml protein solution (1.6 mg/ml 0.05 M Mes, pH 6.5). Dialyze against 0.05 M Mes, pH 6.5 and concentrate to 500 µl (final protein concentration 40 mg/ml).

XAS sample preparation. The XAS sample cell consists of a polyvinylchloride body (29 x 24 x 1 mm³) with a rectangular aperture (15 x 10 mm²), forming the sample chamber (150 µl). Two rectangular pieces (windows) of Kapton (polyimide film) (24 x 18 mm²) were glued on both sides of the cell surrounding the aperture using cyanoacrylate glue (Permacol). The cell was filled with the protein samples through the radial perforations, which were
subsequently sealed with a small drop of glue. The sample was then rapidly frozen in liquid nitrogen to prevent leakages, and stored in a liquid-nitrogen filled Dewar vessel until the XAS measurement.

**XAS measurements.** EXAFS measurements reported here were carried out at the EXAFS station of the European Molecular Biology Laboratory (EMBL) Outstation at the Deutsche Elektronen Synchrotron (DESY) in Hamburg, Germany. The EXAFS station features an order sorting (Si[111] double crystal) monochromator [18], which was set at 50 % of peak intensity to suppress harmonics, a CANBERRA 13 element solid-state fluorescence detector, and an energy calibration device [19]. For fluorescence-mode measurements the detector was placed at an angle of 90° in the horizontal plane with respect to the radiation beam. The cell must be placed under an angle of 45° to the beam to allow fluorescence radiation to be collected onto the detector. The EXAFS scans were acquired around the Zn K-edge (9660 eV) between 9350 and 10359 eV, and the Fe K-edge (7120 eV) between 6900 and (initially) 8000, later 7600 eV. Between 15 to 25 scans per sample were taken. The XANES regions of the scans were compared before averaging to check for any changes in valence state and/or ligand geometry during exposure to X-rays; none were detected. It was found that some of the detectors were hit at the Fe edge by X-ray diffraction peaks (in addition to the fluorescence); this is presumably due to small ice crystals, although addition of a glycerol (up to 20 % v/v) as a cryoprotectant failed to improve the quality of the data. As a result not all detector contributions could be included in the summation, resulting in a lower signal-to-noise ratio than for the Zn EXAFS, and the Fe EXAFS had to be truncated at 475 eV (k = approx. 11 Å⁻¹). The samples were kept at 20 K in the He exchange gas atmosphere of a closed-cycle cryostat during the measurements. During data collection, the storage ring DORIS III was operated at 4.5 GeV in the dedicated mode with ring currents between 140 and 85 mA.
EXAFS data reduction and simulations. Data reduction was carried out with the EMBL Outstation data reduction package [20] including the energy calibration programs CALIB and ROTAX, the averaging program AVERAGE, and the background subtraction program REMOVE. Simulations of the calibrated, averaged, and background-subtracted EXAFS were carried out using default settings (von Barth ground state energy, Hedin-Lundqvist excited state exchange term) in the PC-compatible version (9.272) of the program EXCURVE [21, 22] for the ab initio calculation of phase shift and backscattering factors. As the starting point for our single scattering simulations for Fe and Zn EXAFS we adopted the models of Fig. 1C and D, respectively, that were deliberately simplified and made symmetrical compared to the crystal structures. The parameters of this model (occupancy, distance, and Debye-Waller-type factor for each shell, and the threshold energy ΔEF) were iteratively refined. The significance of each contribution to the fit was assessed from decreases in the values of the fit index and χ² (see Table S1 in Electronic Supplementary Material).

In simulations based on Brookhaven Protein Data Bank (pdb) files, the ligand atoms within 5 Å of each Fe in the ferroxidase site were taken from the relevant pdb files (1nfv for isolated/reoxidised (A), and 1nf4 for reduced (B)) and grouped in two clusters, one around each of the iron ions FeA and FeB which were assumed to contribute equally to the EXAFS. The atoms of the amino acid side chains were grouped in units; contrary to the simulations based on the simplified model described above, the multiple scattering effects within such units were also calculated, using the default (small atom approach [23]) settings of EXCURVE. Before refinement, the atoms for the shells in the pdb files up to 3.4 Å were also grouped around average values, with values for the Debye-Waller-type factors that increased with distance, matching the intensity of the peak in the phase-corrected Fourier transform.
Iterative refinement of the simulation was carried out with just a few parameters: the threshold energy $\Delta E_F$: distances for a close and more remote subshell of the main shell, and for the shells at approximately 3.0 and 3.4 Å. Attempts to include the Debye-Waller-type factors in these refinements led to situations where these refined to lower values for shells further away from the absorber and were therefore abandoned. In view of the good agreement already existing in the simulation based on the non-adjusted pdb file, the outer shells were not adjusted or refined.

**Results and Discussion**

*Bacterioferritin (DdBfr)*

*XANES and EXAFS.* With its relatively stable ferroxidase site which has been crystallographically characterised in various states, *DdBfr* serves as an anchor point for this study. Contrary to the native *DdBfr*, which contains haem and a fully loaded di-iron ferroxidase centre, the recombinant *DdBfr* studied in this paper is obtained free of haem and iron. This recombinant *DdBfr* was reconstituted (see Materials and Methods for details) with Fe(II) and oxidised to give samples corresponding to the states referred to as oxidised, reduced, and oxidised/Chelex® treated. In the crystallographic study of native *DdBfr* [9], three different *DdBfr* structures were determined, viz. structures A (as isolated, anaerobically purified and aerobically crystallised), B (crystal reduced with dithionite), and C (solution reduced with dithionite, cleaned by gel filtration, and anaerobically crystallised). As shown in Fig. 2, the Fe XANES edge position (7121.4 eV) of bis-Fe(II) *DdBfr* (reduced) was significantly shifted (by 1.3 eV to 7222.7 eV, Figure 2) upon exposure to air (oxidized *DdBfr*), confirming the change in valence state expected for iron oxidation. Using the
absolute energy calibration of the EMBL EXAFS station [19], the edge energies are not
subject to accidental shifts due to \textit{i.e.} variations in the angle at which the X-ray beam hits the
monochromator crystals; they are also in line with literature data on ferritin in various
oxidation states [24]. No reduction of the metal ion in the metalloprotein samples by X-rays
was detectable, because neither a shift in the edge position nor a lowering of the intensity of
the first maximum in the X-ray absorption as a function of exposure time was observed.

\textit{- - - FIGURE 2 - - -}

It was of course of interest to see if the difference in edge position and hence in
valence state was in any way reflected in the bond distances and occupancies to be derived
from the EXAFS. The EXAFS is an average of contributions of the Fe ions in the left and
right ferroxidase sites, and even though most Fe-O/N distances are probably similar, it is only
the Fe-Fe distance that is the same for both. In our initial simulations we adopted a model
(Fig. 1C) that was deliberately simplified (including substitution of the single imidazole N
ligand donor atom per Fe, which is not resolved from the other first shell ligands, by another
O, which is virtually indistinguishable in EXAFS) and made symmetrical compared to the
crystal structure (Fig. 1B); this model has a number of independent parameters to be refined
iteratively that is as low as possible, but it does account for all the resolved shells in such a
way that the results can still be related to a molecular model for the ferroxidase site. One
would normally not expect to detect the non-coordinating oxygen in a carboxylate group; in
the model of Fig. 1C this is more likely because of its rigidity (a ring of 2 carboxylate and 2
metal ions holding eachother in place, leading to more correlated thermal motion, and hence
lower values for the Debye-Waller-type factors, for all the absorber-backscatterer pairs
involved) and geometry (the Fe-O-C and Fe-O-O angles are approx. 140 and 125°,
respectively, which makes enhancement of the contribution of the remote atom by multiple scattering effects more likely). It has to be noted that simulations based on models such as in Fig. 1C, where atoms are not grouped in a multiple scattering unit, and only single scattering contributions are considered, might lead to relatively large errors in the outer shell occupancies and even distances due to the neglect of these effects. Taking this approach, simulations (Figure 3, Table 1) using EXCURVE [21, 22] show that the average Fe-O/N ligand distance changes from 2.02 Å for the reduced to 1.99 Å for the oxidised sample, in line with the shorter ionic radius expected for the Fe(III) ion compared to the Fe(II). The Fe-Fe distances for oxidised and reduced protein obtained from these refined simulations are much more similar than those in the crystal structure, viz. EXAFS 3.810 Å for reduced vs. 3.882 Å for oxidised (crystallography: structure A - 3.99 Å vs. structure B - 3.71 Å). It should be noted, however, that the effect of inclusion of the Fe-Fe contribution on the fit index is small (see Table S1, Electronic Supplementary Material), and that satisfactory refined simulations with fit indices only marginally higher than those for the simulations that are shown here can be obtained when the Fe and remote O shells are interchanged, resulting in Fe-Fe distances of approx. 3.4 Å, which is significantly shorter than the crystallographic values. Such a model for the simulations of the ferroxidase EXAFS would be more difficult to link with any of the models B and C in Figure 1, however. There are examples of high-resolution small molecule crystal structures that feature Fe-Fe distances similar to the ones found in the Dd/Bfr crystal structure and in our final EXAFS simulations, such as that of (μ2-acetato)-(μ2-m-xylylenediamine bis(Kemp’s triacid) imido)-bis(N-methylimidazol-3-yl)-tris(methanol)-di-iron(ii) tetrafluoroborate (CSD code REKFUI, Fe-Fe 3.827 Å) [25].

For the oxidised/Chelex®-treated Dd/Bfr sample the situation is different: an even shorter average Fe-ligand distance (1.97 Å, compatible with five- to six-
according to bond valence sum analysis, as discussed below) is observed, along with an
unambiguous Fe-Fe contribution (see Table S1, Electronic Supplementary Material), with
increased (1.5) occupancy, at 3.39 Å. The crystal structure of the wild type DdBfr that was
aerobically crystallised (structure C, the cycled-oxidised, or reduced-then oxidised structure)
shows that only one of the Fe sites in the ferroxidase site, the FeB (right) site, is occupied, in
which case no Fe-Fe distance is expected to be detectable by EXAFS. The distance of 3.4 Å is
more characteristic of mineralization of Fe together with O in the ferritin core, cf. the results
for fully loaded horse spleen ferritin [12] (6 O at 1.96 Å, 1.1 Fe at 3.43 Å), and the spectra
resemble those of [26] which are simulated with 5 O at 1.95 Å and two iron contributions (3.7
at 3.00, and 2.0 at 3.53 Å). A distance of 3.38 Å for a Fe-Fe contribution, along with 5-6 N/O
at 1.96 Å, has also been observed for horse spleen apoferritin reconstituted with 2
Fe(III)/subunit [11], and most likely represents a protein-bound polynuclear Fe-O cluster
there. The EXAFS result for oxidised/Chelex®-treated DdBfr, including the increased
occupancies of the remote O and Fe shells, points to the presence of biomineralized Fe in the
sample. It would imply that following oxidation part of the iron leaks from the ferroxidase
site, leaving only the FeB site occupied, and forms Fe-O clusters so small and so irregular that
they escaped detection in the crystallographic study of the wild type [9]. Clearly, of the three
states of DdBfr studied here, the oxidised/Chelex®-treated sample is least interesting in terms
of direct comparison of EXAFS and crystallography results, but the EXAFS does give an
indication of where the Fe supposed to be present in the FeA site in structure C of the
crystallographic study may have gone.
Comparison with Crystal Structure. The crystal structures of the as-isolated (A) and reduced (B) wild-type $Dd$Bfr [9] show little symmetry in the Fe coordination (Table 2), and no indication of a difference in ionic radius between the Fe(II) and Fe(III) states. It is of interest to compare the EXAFS results for the oxidised and reduced $Dd$Bfr samples with what are most likely to be their crystallographic analogues, the as-isolated structure A and the reduced structure B, respectively. The average ligand distance determined by EXAFS (Table 1) for oxidised $Dd$Bfr is shorter by 0.25 Å, that for reduced $Dd$Bfr by 0.21 Å. The difference in the average ligand distances is larger than can be explained by the accepted errors for either technique (± 0.02 Å for EXAFS, ± 0.14 Å for crystallography). From the crystallographic data for the $Dd$Bfr ferroxidase site the static disorder $\sigma_{\text{static}}$ can be calculated on the basis of the deviations of the Fe-ligand distances from the average value (see Table 2) giving values of 0.15 Å for the as-isolated form A, and 0.14 Å for the reduced form B. The Debye-Waller-type factor $\sigma$ (or EXAFS Debye-Waller factor, refined as $2\sigma^2$ in EXCURVE simulations) is the sum of contributions representing static ($\sigma_{\text{static}}$) and thermal ($\sigma_{\text{thermal}}$) disorder and can therefore be considered to indicate the maximum possible static disorder or variance, assuming that there is no thermal disorder at 20 K. The Debye-Waller-type factors refine to 0.09 and 0.10 Å for oxidised and reduced, respectively. These values are significantly lower than the crystallographically derived $\sigma_{\text{static}}$, which is a strong indication that the ferroxidase site is probably more symmetrical, with less spread in the Fe-ligand distances, than the crystallographic model suggests. The case is comparable, albeit more complicated, to that of an early biological EXAFS study on rubredoxin, where the possibility that rubredoxin had both relatively long and short Fe-S distances was rejected [27, 28].

--- TABLE 2 ---
The longer average distance for the crystal structure is caused in part by the inclusion of both oxygens of bidentate Glu residues, and it is possible that the more remote oxygen does not contribute to the EXAFS. In the initial EXAFS simulations, the occupancy of the main shell (coordination number) refines to values around 5, but because of their strong correlation with the Debye-Waller-type factor the error of coordination numbers can be as high as 30%: a refined value of 5 could therefore still be 4 or 6 in reality. Bond valence sum analysis (BVSA, [29]) offers an approach to use the accurate determination of bond lengths to get information about the coordination numbers. For the application to ferritin, we have neglected the contribution of the single histidine N per iron site and substituted it by another O, keeping in mind that a typical Fe-N distance is longer than a Fe-O distance by 0.07 Å for Fe(II) and 0.1 Å for Fe(III). For the Fe(II) (reduced) protein the average Fe(II)-O ligand distance of 2.02 Å found by EXAFS is in between those characteristic of four- (1.99 Å) and five-coordination (2.07 Å). This could point to the presence of one four- and one five-coordinated site, which would imply that the longest Fe-ligand contacts found in the crystallographic structure B (reduced), viz. the water oxygen in the left (FeA) site and the remote E99-carboxylate oxygen in the right (FeB) site, both at 2.46 Å, do not contribute to the EXAFS. For the oxidised protein, the average Fe(III)-O ligand distance of 1.99 Å found by EXAFS is in between those characteristic of five- (1.95 Å) and six-coordination (2.02 Å). This means that all the ligands found in the oxidised crystallographic structure A (as-isolated) probably contribute to the EXAFS, even the remote E99 carboxylate oxygen and H135 nitrogen found at 2.52 and 2.57 Å, respectively. There is also the possibility that coordination positions that are apparently empty in the crystal structure are taken up by small ligands, such as water, molecular oxygen, or peroxide, at a relatively short distance, that have gone undetected in the crystal structure. For the as-isolated (oxidised) DdBfr (A) this should certainly be considered, as there is some electron density on the site of the Fe(III) corresponding to the top in Fig. 1B, which can be
fitted with water and/or (per)oxo-bridges, but not unequivocally, presumably because of the
presence of a mixture of states. Recently [30] as-isolated \(Dd\)Bfr has been reinvestigated by
crystallography under circumstances where radiation damage is minimised, and an end-on
peroxo ligand to Fe\(B\) could be tentatively identified. It is worth noting that such a non-protein
ligand would be compatible with the occupancy determined for oxidised \(Dd\)Bfr by EXAFS, as
well as with further considerations on the coordination number based on BVSA.

We find that the EXAFS ligand distances are in good agreement with what is expected
for five- or six-coordination according to BVSA [29], especially for the Fe(III) valence state.
Moreover, the crystallographic distances are much higher than those considered typical for Fe
on the basis of a survey of high-resolution crystal structures [31], which are 2.01 \(\text{Å}\) for Fe-
O(carboxylate) and 2.08 \(\text{Å}\) for Fe-N(histidine). These conclusions inspire confidence in the
EXAFS results. We decided to use the EXAFS simulation program EXCURVE to simulate
EXAFS on the basis of crystallographic coordinates as available from the Brookhaven Protein
Data Base, a so-called pdb file, in order to see where the main deviations were and by which
adjustments better agreement between EXAFS and crystallography results might be reached.
In these simulations, the ligand atoms within 5 \(\text{Å}\) of each Fe in the ferroxidase site were taken
from the relevant .pdb files (1nfv for as-isolated (A) and 1nf4 for reduced (B), applied to the
oxidised and reduced \(Dd\)Bfr EXAFS results, respectively) and grouped in two clusters, one
around each of the iron ions Fe\(A\) and Fe\(B\) which were assumed to contribute equally to the
EXAFS. The atoms of the amino acid side chains were grouped in units; contrary to the
simulations based on the simplified model described above, multiple scattering effects within
such units were also calculated.
As expected after our simulations based on the symmetrised model in Fig. 1C, the initial agreement between the experimental EXAFS and the simulation based on the coordinates from the Protein Data Base was very poor (see trace (i) in Figure 4 for reduced \textit{DdBfr}, and Figure S1 in the Supplementary Material). Not only were the Fe-ligand distances overestimated, but in addition the static disorder or variance in the Fe-ligand distances was larger than expected. On the other hand, the agreement between theory and experiment for the outer shells was already very good, considering that all Debye-Waller-type factors had been arbitrarily set to 0.010 Å\(^2\), and a value for the threshold energy \(\Delta E_F\) had been chosen, not refined. The agreement for the main shells was improved considerably by setting the average Fe-ligand distance of this shell to the value arrived at in the preliminary simulations presented in Figure 3 and Table 1, and adjusting the Debye-Waller-type factor for this shell by hand. Obviously this still resulted in poor agreement with the crystallographic distances, as discussed above (average crystallographic distance underestimated by 0.21-0.25 Å). It was noted that the values for the Debye-Waller-type factors used in the simulation for the first shell (0.017 Å\(^2\) for oxidized, 0.022 Å\(^2\) for reduced) were much higher than the value of 0.010 Å\(^2\) that was arbitrarily chosen for the outer shells and found to give good agreement there. This is an artificial situation as the values for the Debye-Waller-type values are supposed to go up with the distance to the central absorber, not down. It was therefore decided to split the main shell into two subshells with lower values for the Debye-Waller-type factors. Detailed inspection of the distances in Table 2 revealed that the oxygens of the bridging glutamate ligands were found at relatively short distances (2.02-2.16 Å, compared to the average of 2.23 Å) from the iron ions in the crystallographic study. These (2 per Fe) were selected to be at a relatively short distances (1.96 Å for oxidised, 1.99 Å for reduced), whereas the others were put at larger distances (2.02 Å for oxidised, 2.05 Å for reduced), in such a way that the resulting average ligand distance was close to that obtained from the EXAFS simulations.
based on a simplified model (Table 1, see Materials and Methods for more details). These adjustments resulted in a significant improvement of the agreement between simulated and experimental EXAFS as can be seen from traces (ii) in Figure 4 (and Figure S1 in the Supplementary Material). The agreement could be even further improved by iterative refinement of the $\Delta$EF and distance parameters (not the Debye-Waller-type factors) resulting in the satisfactory fits of traces (iii) in Figure 4 (and Figure S1). It can be seen from a comparison of the EXAFS parameters obtained in this way, given in italic in Table 2, that the distances to the bridging glutamate oxygens need to be shortened by only approx. 0.1 Å, and to the other atoms, except the very remote ones, by only 0.15 Å to get agreement. This is a far less drastic shift than the 0.2-0.25 Å difference in the average values and one that is almost within the accepted error for the crystallographic distances. The adjusted distances resulting from the EXAFS are given together with the crystallographic values in italic in Table 2, and a graphical representation of the adjustment for the reduced $Dd\text{Bfr}$, based on .pdb file 1nf4 [9], is given in Figure 5. It would have been of interest to investigate whether the crystallographic data of the $Dd\text{Bfr}$ crystal structure are better fitted by a model based on the metal-ligand distances obtained by EXAFS. A practical problem is the large number of protein monomers (16) comprised in the asymmetric unit, which means that the coordinates of the ligands of 32 Fe atoms would have to be edited before the structure could be refined again. Another problem is that at the current resolution (around 2 Å for the $Dd\text{Bfr}$ structures [9]), the apparent electron density around the metal ions is disturbed by termination effects in the Fourier transformation (Fourier ripple effects, see [32] for an early discussion of the case of a dinuclear Fe protein). There are examples where metalloprotein structures have been improved by EXAFS studies but they are usually based on new crystallographic data with a higher resolution (see [33] for examples).
The application of the procedure described above for the reduced and oxidised DdBfr to the sample corresponding to the Chelex®-treated DdBfr using coordinates of the structure from the crystallographic study [9] that most corresponds to it, the aerobically crystallised, cycled-oxidised (reduced, then oxidised) DdBfr (structure C, .pdb file 1nf6), was expected to be problematic. The simulations based on the simplified model (Figure 3, Table 1) had detected the presence of a strong Fe-Fe contribution at 3.4 Å, which was not expected on the basis of the crystal structure [9], where only FeB is occupied. Indeed it was found necessary to add an Fe contribution at 3.4 Å in the EXAFS simulation based on .pdb file 1nf6. As discussed above, this Fe-Fe distance is reminiscent of that found in polynuclear Fe-O clusters such as found in the core of ferritin loaded with Fe; the simulation result confirms that at least in the EXAFS sample the Fe is not only present in the ferroxidase site, but also mainly in such (bio)minerals. The fact that the EXAFS spectrum at best partially represents the FeB site in .pdb file 1nf6 makes it difficult to investigate the role of one or perhaps even two water ligands to this iron.

**Human Ferritin H chain (rHuHF)**

**Zn-loaded rHuHF variants.** A number of Human ferritin H-chain mutants have been studied by crystallography [8] using Zn(II) ions as redox-invariant analogues of Fe(II), and this approach to study ferritin with other metal ions such as Mn has also been taken for X-ray absorption spectroscopic studies [34]. The Zn K edge spectra of the rHuHF mutants incubated with 2 molar eq. of Zn(II) differs mainly in the intensity of the so-called white line, the first maximum in the X-ray absorption spectrum at 9670 eV (white line, Fig. 6). For a coherent
coordination sphere an increase in intensity in the white line can be attributed to a higher
coordination number [35]. Comparing the white line intensities with those of model
compounds of known coordination (Figure 6) [36, 37], it can be concluded that rHuHF-
E107D and rHuHF-E107/E27D mutants, which are indistinguishable, are close to the four-
coordinated model compounds. The intensities of the white lines of the wild type (K86Q) and
rHuHF-E27D mutant, which are also indistinguishable, are between those characteristic for
four- and five-coordination, possibly indicating the presence of one four- and one five-
coordinate site.

- - - FIGURE 6 - - -

By analogy to the model for the bacterioferritin ferroxidase site (see above, Fig. 1C), a
symmetrised model was constructed for the dinuclear Zn-loaded HuHF ferroxidase site (Fig.
1D). In one aspect this model is a larger simplification compared to the structure known to
exist in the protein crystal structure, viz. HuHF contains only one glutamate bridge (Fig. 1A)
whereas bacterioferritin (Fig. 1B) and the model (Fig. 1D) contain two. On the other hand, the
simplification of the substitution of the imidazole by another oxygen is more justified, since
bacterioferritin contains 2 His ligands (1 His ligand/metal), whereas the HuHF has only one
(to FeA). Simulations of the Zn-rHuHF EXAFS spectra (Figure 7, Table 3) reveal strong
similarities. The spectra show Zn-O (probably carboxylate O) contributions around 2 Å, Zn-C
(again carboxylate) at 3 Å, and Zn-Zn distances at 4 Å. The observed Zn-Zn distances are a
little shorter than the expected distances of remote imidazole ring atoms (4.2 Å). However,
attempts to simulate the spectrum with rigid imidazoles failed because a remote ring atom at
4.0 Å would imply a ligand donor atom at 1.8 Å; this is not compatible with the data and
moreover chemically unrealistic. It must be concluded that the single imidazole per metal
atom in the HuHF ferroxidase site does not contribute significantly to the EXAFS. This is somewhat unexpected, but it must be noted that examples where even three imidazole ligands to an iron atom do not give a detectable outer shell contribution to the EXAFS have been reported [38]. The intensity of those EXAFS features has also been found to depend on the orientation of the imidazole ligand with respect to the metal-donor atom bond [36].

--- FIGURE 7, TABLE 3 ---

The first-shell distances (2.00-2.01 Å) are in good agreement with those expected for a four-coordinate Zn-O complex according to BVSA [29], viz. 2.03 Å, and with the Zn-O(carboxylate) distance of 2.04 Å found in the survey of high-resolution crystal structures [31], especially when the possible presence of one shorter Zn-N(His) (1.96 in BVSA, 2.00 Å in the survey) is taken into account. The lack of significant differences is surprising in view of the effects that might have been expected on the basis of the mutants of the glutamate ligands on either site of the dinuclear site. However, it should be noted that the geometries, in particular the Zn-Zn distances, of the Zn-occupied ferroxidase sites as determined in the various mutants by crystallography [8] are also rather similar (3.32 Å for “wild type” K86Q, 3.44 Å for E27D, and 3.38 Å for E107D) apart from rHuHF- E27D/E107D, which did not have its ferroxidase site occupied with two Zn ions in the crystal structure. The Zn-Zn distances determined by crystallography [8] are much shorter than those determined by EXAFS in the present study. Additional EXAFS simulations where the Zn and O shells at 4.0 and 3.8 Å are interchanged have almost as good fit indices as the ones presented in Table 3 and Figure 7, but the Zn-Zn distance from EXAFS is under no circumstances shorter than 3.8 Å. We note that there are examples of crystal structures of synthetic dinuclear Zn complexes that feature Zn-Zn distances in this range, such as bis(μ2-acetato)-bis(ethyl 2-cyano-3-[(2,6-di-
isopropylphenyl)amino]-N-(2,6-diethylphenyl)prop-2-enimidoato)-di-zinc (CSD code DEQTEZ, Zn-Zn 3.963 Å) [39] and bis((μ₂-acetato-O,O’)-(2-(2-(pyrrolidyl)ethyl)-1,3,4,5-tetramethyl-cyclopentadienyl))-di-zinc (CSD code XOJSEU, Zn-Zn 3.823 Å) [40]. We conclude that the most important factor that determines the Zn-Zn distance is the bridging glutamate (E62), and that moving the carboxylates of E27 and/or E107 has little effect. Crystallography shows that the Zn sites are three- or four-coordinated, with Zn-ligand distances for “wild-type” (K86Q, average 2.16 Å) and rHuHF-E27D (2.10 Å) that are longer than those determined by EXAFS, although not as much longer as those in the DdBfr structure. For “wild type” and this mutant the agreement between EXAFS and crystal structure rHuHF is quite good if one moves each Zn ion 0.10-0.15 Å towards the glutamate ligands at either end of the ferroxidase site, elongating the Zn-Zn distance correspondingly. For rHuHF-E107D the distance of the D107 to the Zn ion to the right is much longer (3.2 Å) than the EXAFS distances, and probably small (water) ligands not observed in the crystal structure complete the coordination sphere for this ion. For rHuHF-E27D/E107D there is no agreement between EXAFS and crystallography, and one must conclude that the soaking of the crystals with Zn and the preparation of the Zn derivative in solution by adding two equivalents of the metal have resulted in samples with different metal loading. A more detailed comparison of the EXAFS and crystallographic results such as discussed for DdBfr is hampered by the problem that in the rHuHF crystal structures, the Zn(II) ions have never more than three protein ligands (in one case – ZnB in rHuHF-E107D – only one) and that only few of the non-protein ligands have been localised, whereas the EXAFS clearly indicates coordination numbers of at least four.

**Fe-loaded rHuHF variants.** Samples were incubated with iron under transiently anaerobic conditions under an argon atmosphere to favour the homogeneity of iron.
distribution; by maintaining iron in the Fe(II) form, we anticipated that, as in the case of
native *Dd*Bfr [9] we would restrict iron uptake to only two iron atoms into each ferroxidase
centre. Controlled oxidation was then allowed to proceed before the sample was flash-frozen
and X-ray absorption data were collected on the frozen solution. In this way we expected to
be able to monitor any oxidation of the iron ions, as well as their possible migration from the
ferroxidase centre to the nucleation site.

The edge spectra in Figure 2 show that the edge position of *rHu*HF-E27D/E107D
mutant sample is close (7121.8 eV) to that of the reduced *Dd*Bfr (7121.4 eV) implying that it
has retained significant Fe(II) character (cf. [24]. The “wild type” *rHu*HF (K86Q) edge is at
7122.5 eV and close to that of the oxidised *Dd*Bfr, implying a significant degree of oxidation
(and perhaps mineralization). The bis-Fe complexes of the mutants in which only a single
glutamate was changed to an aspartate, *rHu*HF-E27D and *rHu*HF-E107D, were found at
intermediate energies. For *rHu*HF and *rHu*HF-E107D, the fast oxidation and its total absence,
respectively, are in line with their observed relative reactivities in the accumulation of Fe(III)
ions upon addition of Fe(II) to the apoprotein under aerobic conditions [8]. Based on these
measurements, *rHu*HF-E27D was expected to be even more easily oxidized than the wild
type, whereas *rHu*HF-E27D/E107D showed no activity at all.

Simulations of the EXAFS spectra on the basis of the symmetrized model (Fig. 1C)
that was also applied to the *Dd*Bfr data were carried out to probe the environment up to 3-4 Å
from the absorbing Fe atom for the presence of a high-Z atom (Fe, see Fig. 8). Some of the Fe
*rHu*HF EXAFS spectra, viz. those for *rHu*HF-E27D/E107D and *rHu*HF-E107D, have lower
signal-to-noise ratio than the others, because fewer detector contributions could be included
(see Materials and Methods); they are nevertheless included here to complement the Zn edge
and EXAFS and Fe edge results. The results of the best simulations are given in Table 4.

Interestingly, none of the observed Fe-O distances is at 2.0 Å or higher such as observed for the Zn analogues or the Fe in reduced DdBfr; all Fe-O distances fall in the range 1.97-1.99 Å, even rHuHF-E107D which appears to have both its Fe as Fe(II) judging from the edge (Fig. 2). For this last mutant, the BVSA indicates that the coordination number most likely correlated with Fe(II)-O distances of 1.99 Å is four; this means that in addition to the expected protein ligands (E62 for both Fe, E27 and H65 to the left, E107 to the right) there is probably at least one small non-protein ligand, possibly the water oxygen already indicated for FeA (Fig. 1A). For the others it is in between five and six, assuming considerable or complete conversion to Fe(III).

For rHuHF-E27D/E107D and rHuHF-E107D very weak Fe-Fe contributions at respectively 3.96 and 3.12 Å were detected, which have to be considered insignificant in view of the noise in the data; the most important conclusion from these simulations is that there are no strong Fe-Fe contributions in the 3.4 Å range. Such contributions were very strongly present in the data for rHuHF and the E27D mutant. We note that these distances are much shorter than those observed for DdBfr and Zn-loaded rHuHF but in the same range as those found with crystallography for Zn-HuHf; however, in the Zn EXAFS such short distances were not detected. Imidazole contributions were not required to simulate any of the rHuHF Fe EXAFS, in line with the observations for the Zn HuHF analogues where there is also only 0.5 imidazole/metal expected. It rather looks like the presence of relatively short Fe-Fe distances for the wild type and the E27D mutant points to the presence of a significant amount of mineralized Fe in those samples. This is also consistent with the relatively high occupancies of the Fe-O shells and the Fe-O distances which are a little bit too short to be all Fe-O(carboxylate) distances when compared to the distance value of 2.01 Å given in the
metalloprotein crystal structure survey [31]. The observation of polynuclear Fe-O cluster formation upon addition of Fe(II) ions to apoferritin is consistent with literature results [11, 41].

--- FIGURE 8, TABLE 4 ---

Concluding Remarks

The X-ray absorption near edge region of the spectrum (XANES) allows the oxidation state of the Fe ions to be assessed, and reflects the oxidation state of the DdBfr as well as the different reactivities of rHuHF mutants. In the case of rHuHF reconstituted with Zn, information on coordination number and geometry is derived from the XANES. Simulations of the Extended X-ray absorption fine structure (EXAFS) have yielded accurate geometric information that represents an important refinement of the crystal structure, adjusting most metal-ligand bonds to shorter distances and allowing differences between the Fe(II) and Fe(III) states that are consistent with the ionic radii to be established. Moreover, the results give a clue about the state of Fe in the aerobically crystallised/cycled-oxidised form of the DdBfr, where the crystal structure (C) shows the dinuclear site to be only half-occupied; the EXAFS of the analogous oxidised/Chelex®-treated sample provides evidence that the other iron is used in a mineralization process. In the case of rHuHF the complexes with the redox-inert Zn reveal a surprising similarity among the variants. This is in line with the crystal structures of 3 mutants, although the metal-ligand and metal-metal distances found with EXAFS are shorter and longer, respectively. The Zn results indicate that the metal-metal distances are not much affected by the mutations of E27 and/or E107 to aspartates. This means that the observed differences in ferroxidase reactivity must be explained by the effect
of the mutations on other parameters not assessed in this EXAFS study, such as the affinity of
each of the sites A and B for the metal ion, and the subsequent reactivity towards dioxygen. In
spite of the apparent rigidity of the dinuclear site the rHuHF complexes show a variation in
reactivity that is reflected in the Fe oxidation states and coordination geometries. As in the
case of the oxidised DdBfr (C), considerable mineralization has occurred for the wild type and
the most reactive mutant (rHuHF-E27D), resulting in EXAFS-detectable Fe-Fe distances of
3.4 Å.

Acknowledgements

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Electronic Supplementary Material available: Figures of k³-weighted EXAFS and
corresponding phase-corrected Fourier transform of oxidised, reduced, and Chelex®-treated
DdBfr, with simulations based on the .pdb files of the corresponding crystal structures. Table
showing the effect of omission of minor shell contributions to the EXAFS simulations on fit
index and χ².

Figure Captions
Figure 1. Schematic representation of ferroxidase site.

(a) Human H ferritin, adapted from ref. [8] (E61 is conserved and was implied in metal
coordination in early crystallographic studies [5, 6]). (b) Oxidised Desulfovibrio desulfuricans
bacterioferritin, adapted from ref. [9] structure A (E99 is asymmetric bidentate in the reduced
structure B, and monodentate in the aerobically crystallised/cycled-oxidised structure C). (c)
and (d), starting models for the EXAFS simulations of Fe- and Zn-loaded ferritins,
respectively, with the numbers in red bold indicating the distance to the red bold metal atom.

Figure 2. Fe K Edge of Fe-loaded ferritins, from low to high energy: blue, rHuHf-E107D;
green, reduced DdBfr; orange, rHuHf-E27D/E107D; brown, rHuHf-E27D; grey, “wild type”
rHuHf (K86Q); turquoise, Chelex®-treated DdBfr; red, oxidised DdBfr.

Figure 3. k²-weighted Fe K EXAFS (left panel) and corresponding phase-corrected Fourier
transform (right) of Desulfovibrio desulfuricans bacterioferritin (DdBfr). Coloured traces,
experimental: turquoise, Chelex®-treated; red, oxidised; green, reduced; black thin solid
traces, corresponding simulations with the parameters listed in Table 1.

Figure 4. k²-weighted EXAFS (left panels) and corresponding phase-corrected Fourier
transform (right) of reduced Desulfovibrio desulfuricans bacterioferritin (DdBfr). (i) EXAFS
simulation on the basis of the .pdb file related to the crystal structure of reduced wild type
DdBfr [9], 1nf4 (distances in Table 2); (ii) major shells adjusted to the average EXAFS value
found in the initial simulation given in trace (iv), Figure 3 and Table 1; (iii) simulation
obtained by iterative refinement of the ΔEF and distance values, starting from the adjustment
in (ii), resulting in the distances given in italic in Table 2; (iv) simulation based on the
simplified model in Fig. 1c, refining to the parameters in Table 1.
Figure 5. Ferroxidase site in reduced DdBfr with Fe-ligand distances in Å: A) as determined by crystallography [9], B) adjusted to give agreement with the EXAFS. Colour code: grey, carbon, red, oxygen; blue, nitrogen; orange, Fe; hydrogens are omitted for clarity. Green and blue distance values refer to FeA (left Fe ion) and FeB (right), respectively.

Figure 6. Zn K edge of Zn-loaded rHuHF (bold: blue rHuHF-E107D, colliding with orange: rHuHF-E27D/E107D; violet: rHuHF-E27D, colliding with green: “wild type” rHuHF (K86Q)) and Zn complexes (thin lines: light green, tetrakis(imidazole)Zn(II) diperchlorate (Zn(Im)₄, tetrahedral 4-c.); orange, bis(acetato)bis(imidazole)Zn(II) (ZnIm₂Ac₂, near tetrahedral 4-c.); red, zinc oxide (ZnO, tetrahedral 4-c.); dark green, bis(L-histidino)Zn(II) dehydrate (Zn(His)₂, tetrahedral 4-c.); turquoise, bis[(imidazol-2-yl-thiophen-2-yl)methanol]zinc(II) sulphate hydrate (Zn(ImThOH)₂, 5-c.); pink, triaquabis(N-benzoylglycinato)zinc(II) dihydrate (Zn(H₂O)₃(BzGly)₂, 5-c.); brown, tetraaquabis(hydrogenmaleato)zinc(II) (Zn(mal)₂, octahedral 6-c.); see [36, 37] for details of models.

Figure 7. k²-weighted Zn K EXAFS (left panel) and corresponding phase-corrected Fourier transform (right) of rHuHF. Coloured traces, experimental: green, “wild type” rHuHF (K86Q); violet, rHuHF-E27D; orange, rHuHF-E27D/E107D; blue, rHuHF-E107D; black thin solid traces, corresponding simulations with the parameters listed in Table 3.

Figure 8. k²-weighted Fe K EXAFS (left panel) and corresponding phase-corrected Fourier transform (right) of rHuHF. Coloured traces, experimental: green, “wild type” rHuHF (K86Q);
### Tables

Table 1. Refined parameters for the Fe K EXAFS simulations shown in Fig. 3

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a) Distances in Å; Debye-Waller-type factors as 2σ² in parentheses in Å²

b) Energy position at half height (eV)
Table 2. Comparison of Fe-ligand distances (in Å) determined by crystallography (normal, adapted from [9], see Fig. 1B for schematic representation) and adjusted/refined by EXAFS (italic).

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<td></td>
<td></td>
<td></td>
<td>(4) 1.97</td>
</tr>
<tr>
<td>σ</td>
<td>0.06</td>
<td>0.20</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(7) 0.57</td>
</tr>
<tr>
<td>Aver/σ</td>
<td>22.35/10 = 2.235</td>
<td>24.45/11 = 2.223</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>= 2.02</td>
<td>= 2.04</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ EXAFS parameters for oxidised $D_dBfr$ (cf. Fig. S1A, trace iii)

$^b$ EXAFS parameters for reduced $D_dBfr$, cf. Fig. 4 (and Fig. S1B), trace iii, and Fig. 5B

$^c$ EXAFS parameters for Chelex®-treated $D_dBfr$ (cf. Fig. S1C, trace iii)
Table 3. Refined parameters for the Zn K EXAFS simulations shown in Fig. 7

<table>
<thead>
<tr>
<th>Shell: O</th>
<th>rHuHF (K86Q)</th>
<th>rHuHF- E27D</th>
<th>rHuHF- E27D/ E107D</th>
<th>rHuHF- E107D</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.9 @ 2.00 (.019)</td>
<td>4.8 @ 2.00 (.018)</td>
<td>4.7 @ 2.00 (.016)</td>
<td>4.9 @ 2.01 (.020)</td>
<td></td>
</tr>
<tr>
<td>Shell: C</td>
<td>0.5 @ 3.01 (.004)</td>
<td>0.6 @ 2.99 (.001)</td>
<td>0.8 @ 3.03 (.006)</td>
<td>0.6 @ 2.98 (.001)</td>
</tr>
<tr>
<td>Shell: O</td>
<td>5.3 @ 3.79 (.025)</td>
<td>4.9 @ 3.78 (.022)</td>
<td>5.4 @ 3.82 (.023)</td>
<td>3.7 @ 3.82 (.017)</td>
</tr>
<tr>
<td>Shell: Zn</td>
<td>0.8 @ 3.98 (.016)</td>
<td>1.0 @ 3.97 (.013)</td>
<td>2.3 @ 4.00 (.030)</td>
<td>1.3 @ 3.99 (.018)</td>
</tr>
<tr>
<td>ΔEF</td>
<td>-10.3233</td>
<td>-10.3910</td>
<td>-11.6095</td>
<td>-11.9367</td>
</tr>
<tr>
<td>Fit index</td>
<td>0.1059*10^{-3}</td>
<td>0.0901*10^{-3}</td>
<td>0.2297*10^{-3}</td>
<td>0.1588*10^{-3}</td>
</tr>
</tbody>
</table>

*) Distances in Å; Debye-Waller-type factors as 2σ² in parentheses in Å².

Table 4. Refined parameters for the Fe K EXAFS simulations shown in Fig. 8

<table>
<thead>
<tr>
<th>Shell: O</th>
<th>rHuHF (K86Q)</th>
<th>rHuHF- E27D</th>
<th>rHuHF- E27D/ E107D</th>
<th>rHuHF- E107D</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 @ 1.98 (.015)</td>
<td>4.7 @ 1.97 (.016)</td>
<td>5.1 @ 1.98 (.021)</td>
<td>4.7 @ 1.99 (.010)</td>
<td></td>
</tr>
<tr>
<td>Shell: C</td>
<td>1.2 @ 3.03 (.003)</td>
<td>1.7 @ 3.11 (.001)</td>
<td>2.7 @ 3.05 (.005)</td>
<td>2.7 @ 2.93 (.004)</td>
</tr>
<tr>
<td>Shell: O</td>
<td>4.4 @ 3.49 (.003)</td>
<td>4.8 @ 3.55 (.003)</td>
<td>6.3 @ 3.46 (.030)</td>
<td>3.3 @ 3.32 (.003)</td>
</tr>
<tr>
<td>Shell: Fe</td>
<td>1.7 @ 3.38 (.004)</td>
<td>2.1 @ 3.40 (.003)</td>
<td>1.1 @ 3.96 (.023)</td>
<td>1.1 @ 3.12 (.003)</td>
</tr>
<tr>
<td>ΔEF</td>
<td>-3.9150</td>
<td>-3.3515</td>
<td>-3.1847</td>
<td>-2.3904</td>
</tr>
<tr>
<td>eV range</td>
<td>2.5 – 475</td>
<td>2.5 – 475</td>
<td>3 – 472</td>
<td>2.5 – 475</td>
</tr>
<tr>
<td>Fit index</td>
<td>0.1620*10^{-4}</td>
<td>0.2310*10^{-4}</td>
<td>0.4782*10^{-4}</td>
<td>0.6311*10^{-4}</td>
</tr>
<tr>
<td>Edge</td>
<td>7122.5</td>
<td>7121.8</td>
<td>7121.8</td>
<td>7121.0</td>
</tr>
</tbody>
</table>

*) Distances in Å; Debye-Waller-type factors as 2σ² in parentheses in Å².

b) Energy position at half height (eV)
References

12. Macedo S, Romao CV, Mitchell E, Matias PM, Liu MY, Xavier AV, LeGall J,
28. Outstation Hamburg, Germany
8 150
10 36. Feiters MC, Eijkelenboom AP, Nolting HF, Krebs B, van den Ent FM, Plasterk RH,
14 38. Randall CR, Zang Y, True AE, Que L, Jr., Charnock JM, Garner CD, Fujishima Y,
19 503
20
21
Figure 1. Schematic representation of ferroxidase site.

(a) Human H ferritin, adapted from ref. [8] (E61 is conserved and was implied in metal coordination in early crystallographic studies [5, 6]). (b) Oxidised Desulfovibrio desulfuricans bacterioferritin, adapted from ref. [9] structure A (E99 is asymmetric bidentate in the reduced structure B, and monodentate in the aerobically crystallised/cycled-oxidised structure C). (c) and (d), starting models for the EXAFS simulations of Fe- and Zn-loaded ferritins, respectively, with the numbers in red bold indicating the distance to the red bold metal atom.
Figure 2. Fe K Edge of Fe-loaded ferritins, from low to high energy: blue, rHuHf-E107D; green, reduced DdBfr; orange, rHuHf-E27D/E107D; brown, rHuHf-E27D; grey, “wild type” rHuHf (K86Q); turquoise, Chelex®-treated DdBfr; red, oxidised DdBfr.
Figure 3. \(k^2\)-weighted Fe K EXAFS (left panel) and corresponding phase-corrected Fourier transform (right) of *Desulfovibrio desulfuricans* bacterioferritin (DdBfr). Coloured traces, experimental: turquoise, Chelex\textsuperscript{®}-treated; red, oxidised; green, reduced; black thin solid traces, corresponding simulations with the parameters listed in Table 1.
Figure 4. $k^2$-weighted EXAFS (left panels) and corresponding phase-corrected Fourier transform (right) of reduced *Desulfovibrio desulfuricans* bacterioferritin (Dbf). (i) EXAFS simulation on the basis of the .pdb file related to the crystal structure of reduced wild type Dbf [9], 1nf4 (distances in Table 2); (ii) major shells adjusted to the average EXAFS value found in the initial simulation given in trace (iv), Figure 3 and Table 1; (iii) simulation obtained by iterative refinement of the AEF and distance values, starting from the adjustment in (ii), resulting in the distances given in italic in Table 2; (iv) simulation based on the simplified model in Fig. 1c, refining to the parameters in Table 1.
Figure 5. Ferroxidase site in reduced $Dd$Bfr with Fe-ligand distances in Å: A) as determined by crystallography [9], B) adjusted to give agreement with the EXAFS. Colour code: grey, carbon, red, oxygen; blue, nitrogen; orange, Fe; hydrogens are omitted for clarity. Green and blue distance values refer to FeA (left Fe ion) and FeB (right), respectively.
Figure 6. Zn K edge of Zn-loaded rHuHf (bold: blue rHuHf-E107D, colliding with orange: rHuHf-E27D/E107D; violet: rHuHf-E27D, colliding with green: “wild type” rHuHf (K86Q)) and Zn complexes (thin lines: light green, tetrakis(imidazole)Zn(II) diperchlorate (Zn(Im)$_4$, tetrahedral 4-c.;) orange, bis(acetato)bis(imidazole)Zn(II) (ZnIm$_2$Ac$_2$, near tetrahedral 4-c.;) red, zinc oxide (ZnO, tetrahedral 4-c.;) dark green, bis(L-histidino)Zn(II) dehydrate (Zn(His)$_2$, tetrahedral 4-c.;) turquoise, bis[(imidazol-2-yl-thiophen-2-yl)methanol]zinc(II) sulphate hydrate (Zn(ImThOH)$_2$, 5-c.;) pink, triaquabis(N-benzoylglycinato)zinc(II) dihydrate (Zn(H$_3$O)$_3$(BzGly)$_2$, 5-c.;) brown, tetraaquabis(hydrogenmaleato)zinc(II) (Zn(mal)$_2$, octahedral 6-c.;) see [36, 37] for details of models.
Figure 7. \( k^2 \)-weighted Zn K EXAFS (left panel) and corresponding phase-corrected Fourier transform (right) of rHuHf. Coloured traces, experimental: green, “wild type” rHuHf (K86Q); violet, rHuHf-E27D; orange, rHuHf-E27D/E107D; blue, rHuHf-E107D; black thin solid traces, corresponding simulations with the parameters listed in Table 3.
Figure 8. $k^2$-weighted Fe K EXAFS (left panel) and corresponding phase-corrected Fourier transform (right) of rHuHf. Coloured traces, experimental: green, “wild type” rHuHf (K86Q); violet, rHuHf-E27D; orange, rHuHf-E27D/E107D; blue, rHuHf-E107D; black thin solid traces, corresponding simulations with the parameters listed in Table 4.