Supplementary Information

Spectroscopic evidence for the role of a site of the di-iron catalytic center of ferritins in tuning the kinetics of Fe(II) oxidation

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Protein expression, purification, and preparation of apoferritin. Pyrococcus furiosus ferritin (PfFtn) and human H-type ferritin (HuHF) were expressed, purified, and made apo as described previously [1].

Preparation of Fe(II) solution for stopped-flow spectroscopy and freeze-quench. The Fe_2SO_4 salt was first dissolved in anaerobic and acidic (pH < 2) solution of Milli Q. water-HCl. After complete dissolution of Fe(II) salt, the solution was purged with O_2 gas for at least 10 min. This solution was used for stopped-flow spectroscopy or for preparation of freeze-quench samples for EPR and Mössbauer spectroscopy. A 1M solution of ^{57}Fe(II) was prepared anaerobically in dilute sulfuric acid and was diluted in anaerobic Milli Q. water to reach the concentration required for each experiment. Subsequently, the ^{57}Fe(II) solution was purged with O_2 for at least 10 min. For each experiment the concentration of Fe(II) was set to achieve addition of circa 2 Fe(II) per ferritin subunit. This amount of Fe(II) was chosen because addition of higher amounts of ^{57}Fe(II) to ferritin 24-mer may result in complicated Mössbauer spectra. This is because in ferritin Fe(II) is substrate and is converted to Fe(III) products via Fe(III) intermediates whose Mössbauer spectra overlap with those of the Fe(II) substrate and the Fe(III) products. Addition of less than 2 Fe(II) per ferritin subunit might not be sufficient to observe enough intermediates during catalysis.

Stopped-flow UV-visible spectroscopy. A High-Tech scientific PQ/SF-53 preparative quench/stopped-flow instrument was used to measure fast kinetics of Fe(II) oxidation by PfFtn. Measurements were performed at 47 °C with PfFtn or at 10 °C with HuHF . Both protein and Fe(II) solutions were O_2 saturated. To keep the pH at 7.0 after mixing of the protein solution with acidic Fe(II) solution and to prevent the pH to drop to a value of 6.5 or less at which Fe(II) binding to the ferroxidase center is abolished[2], the protein solution was prepared in 1M MOPS buffer, 200mM NaCl, pH 7.0. A pH control experiment by mixing the highly acidic Fe(II) solution with the buffer at a 1:1 ratio was performed to check for any change in the pH. After mixing the Fe(II) and protein solutions spectra were recorded from 300 nm to 740 nm. Final concentration of PfFtn was 4.5 μM (24-mer) and that of HuHF was 2.2 μM (24-mer). Data analysis was performed in IGOR Pro software.

Deriving statistical equations to calculate percentage of each Fe(II)-occupied subunit type using the results of Mössbauer spectroscopy. Based on distribution of Fe(II) among three sites, four Fe(II)-occupied subunit types are possible: ($A^{II}B^{II}C^{0}$)

subunits with Fe(II)-occupied sites A and B but empty site C; ($A^{II}B^{II}C^{II}$) subunits with Fe(II)-occupied sites A, B, and C; ($A^{II}B^{0}C^{II}$) subunits with Fe(II)-occupied sites A and C but empty site B; and ($A^{II}B^{0}C^{0}$) subunits with Fe(II)-occupied site A only. Accordingly we define three variables:

$$X = \frac{\%(A^{II}B^{0}C^{0}) + \%(A^{II}B^{II}C^{0}) + \%(A^{II}B^{0}C^{II}) + \%(A^{II}B^{II}C^{II})}{100} \quad (1)$$

$$Y = \frac{\%(A^{II}B^{II}C^{0}) + \%(A^{II}B^{II}C^{II})}{100} \quad (2)$$

$$Z = \frac{\%(A^{II}B^{0}C^{II}) + \%(A^{II}B^{0}C^{0})}{100} = X - Y \quad (3)$$

We added circa 50 Fe(II) per ferritin 24-mer and using Mössbauer spectroscopy a percentage of this Fe(II) was assigned to sites A or B. X and Y represent the subunits whose site A or site B is filled respectively. Thus, X or Y can be obtained from the percentage of Fe(II) assigned to site A or site B respectively, multiplied by the total amount of Fe(II) added per ferritin 24-mer (n) to obtain the amount of Fe(II) in site A or site B. This value divided by the number of subunits (24) and by 100 will provide the value of X and Y. Therefore for X, Y, and Z we can write:

$$X = \frac{\left(\frac{n \times \%\ Fe(II)\ in\ site\ A}{24\ subunits}\right)}{100} \quad (4)$$

$$Y = \frac{\left(\frac{n \times \%\ Fe(II)\ in\ site\ B}{24\ subunits}\right)}{100} \quad (5)$$

$$Z = X - Y$$

Accordingly, the percentage of ($A^{II}B^{0}C^{II}$) subunits whose site B is empty can be obtained. First the total amount of Fe(II) in site C can be obtained by multiplying the percentage of Fe(II) assigned to site C based on the results of Mössbauer spectroscopy and the number of the total Fe(II) added per ferritin 24-mer (n). This amount is distributed in ($A^{II}B^{0}C^{II}$) subunits and ($A^{II}B^{II}C^{II}$) subunits. Thus, the amount present in ($A^{II}B^{0}C^{II}$) subunits can be obtained by multiplying the total amount of Fe(II) in site C by the value of 'Z'. This value divided by 24-mer will provide the percentage of ($A^{II}B^{0}C^{II}$) subunits. Therefore, for the percentage of ($A^{II}B^{0}C^{II}$) subunits we can write:

$$\%A^{II}B^{0}C^{II} = \frac{n \times \%\, Fe(II)\ in\ site\ C}{24\ subunits} \times (Z) \quad (6)$$

Subsequently, the percentage of ($A^{II}B^{II}C^{II}$) subunits can be obtained. First, the percentage of Fe(II) in site C of ($A^{II}B^{II}C^{II}$) subunits can be obtain by subtracting the percentage of Fe(II) in site C of ($A^{II}B^{0}C^{II}$) subunits from the percentage of the total Fe(II) assigned to site C. This percentage multiplied by the number of Fe(II) added (n) and Y, which represents the sum of ($A^{II}B^{II}C^{II}$) and ($A^{II}B^{II}C^{0}$) subunits, and divided by the number of subunits (24) will give the percentage of ($A^{II}B^{II}C^{II}$) subunits. Thus, we can write:

$$\%A^{II}B^{II}C^{II} = \frac{n \times \left(\%Fe(II)\ in\ site\ C - \%\ of\ Fe(II) in\ site\ C\ of\ A^{II}B^{0}C^{II}\right)}{24\ subunits} \times Y \quad (7)$$

In which the % of Fe(II) in site c of ($A^{II}B^{0}C^{II}$) subunits is:

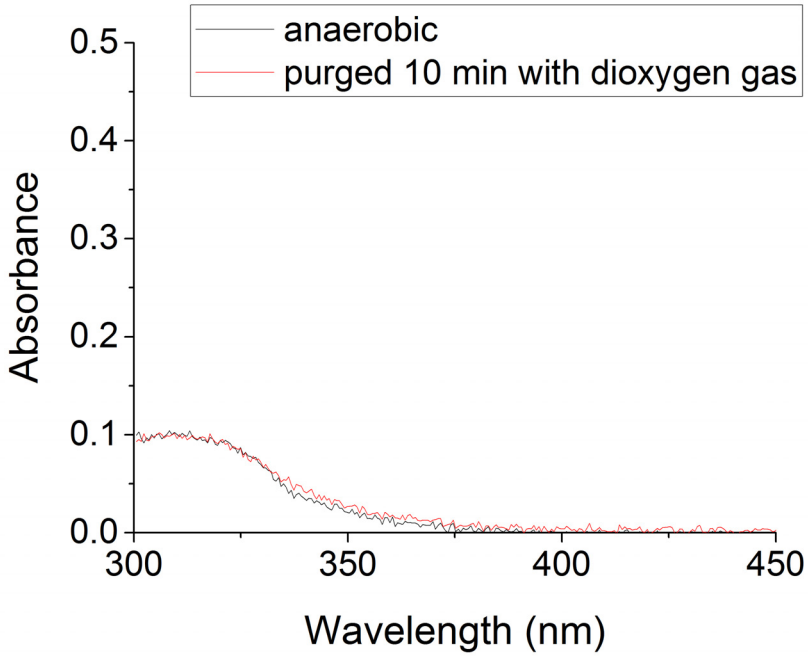
$$\%\ of\ Fe(II) in\ site\ C\ of\ (A^{II}B^{0}C^{II})\ subunits = \frac{\left(\%\ A^{II}B^{0}C^{II}\right) \times 24}{n} \quad (8)$$

Knowing the percentages of ($A^{II}B^{II}C^{II}$) and ($A^{II}B^{0}C^{II}$) subunits, the percentage of ($A^{II}B^{II}C^{0}$) and ($A^{II}B^{0}C^{0}$) subunits can be obtained from equations 2 and 1 respectively.

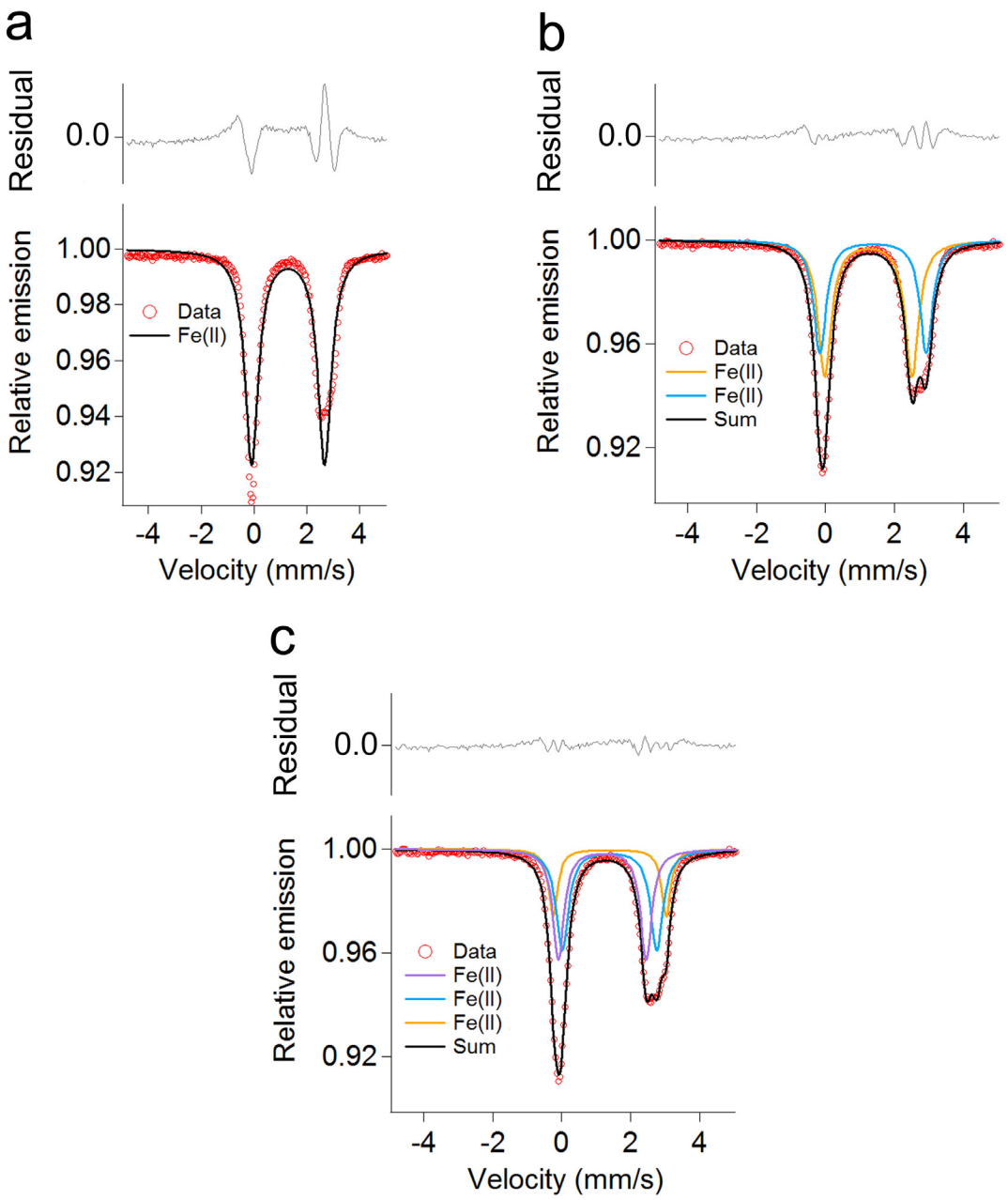
$$\%A^{II}B^{II}C^{0} = (Y \times 100) - \%A^{II}B^{II}C^{II} \quad (9)$$

$$\%A^{II}B^{0}C^{0} = (Z \times 100) - \%A^{II}B^{0}C^{II} \quad (10)$$

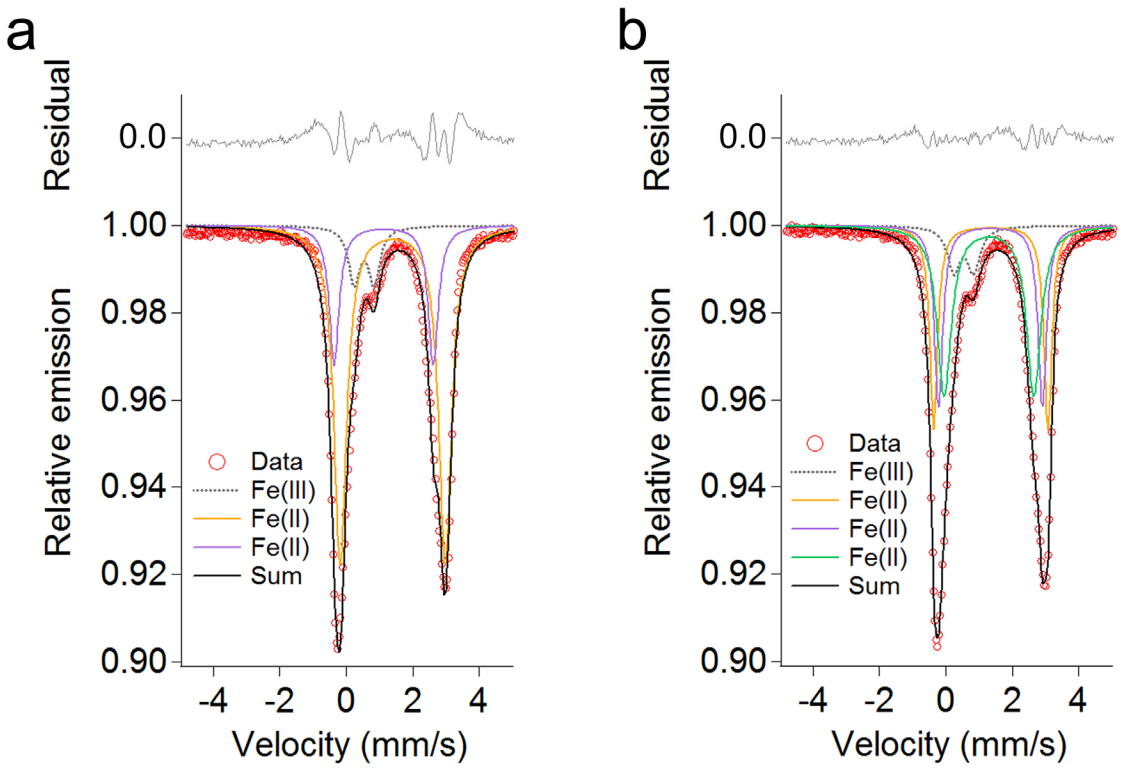
Supplementary Figures



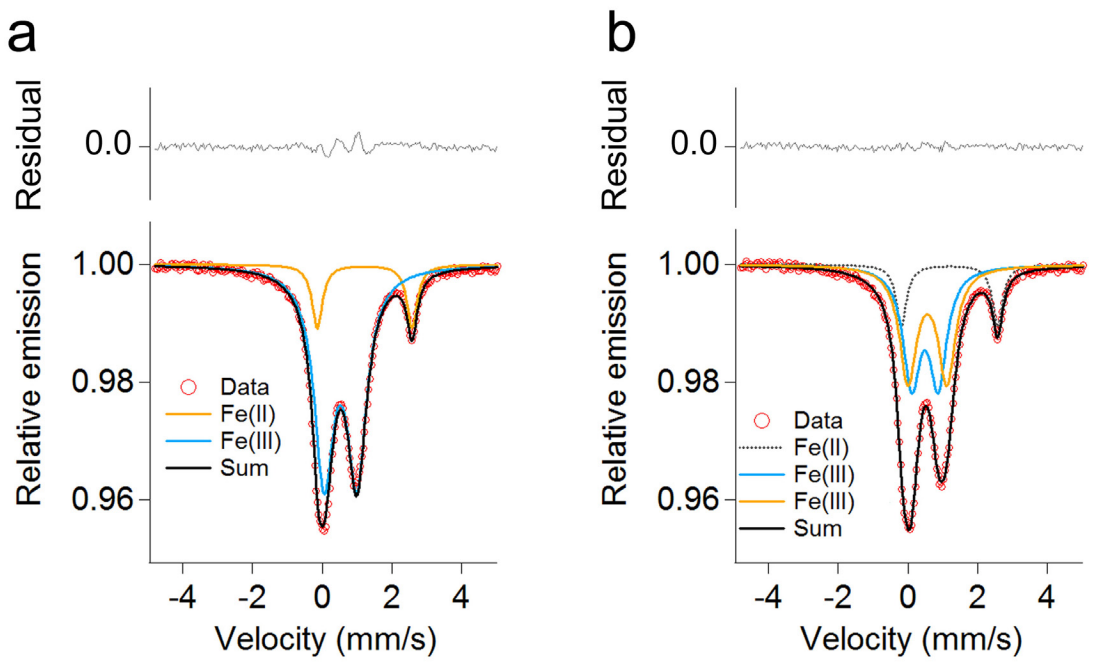
Supplementary Figure 1. Acidic solution of Fe(II) is stable. An acidic solution of Fe(II) (2 mM), pH 2, was purged for 10 min with dioxygen gas. The UV-visible spectra were recorded before purging with dioxygen under anaerobic conditions and after 10 min purging with pure dioxygen. No difference in the absorbance was observed. The small absorbance observed for the Fe(II) solution before and after purging with dioxygen is possibly due to small amounts of dirty Fe(III).



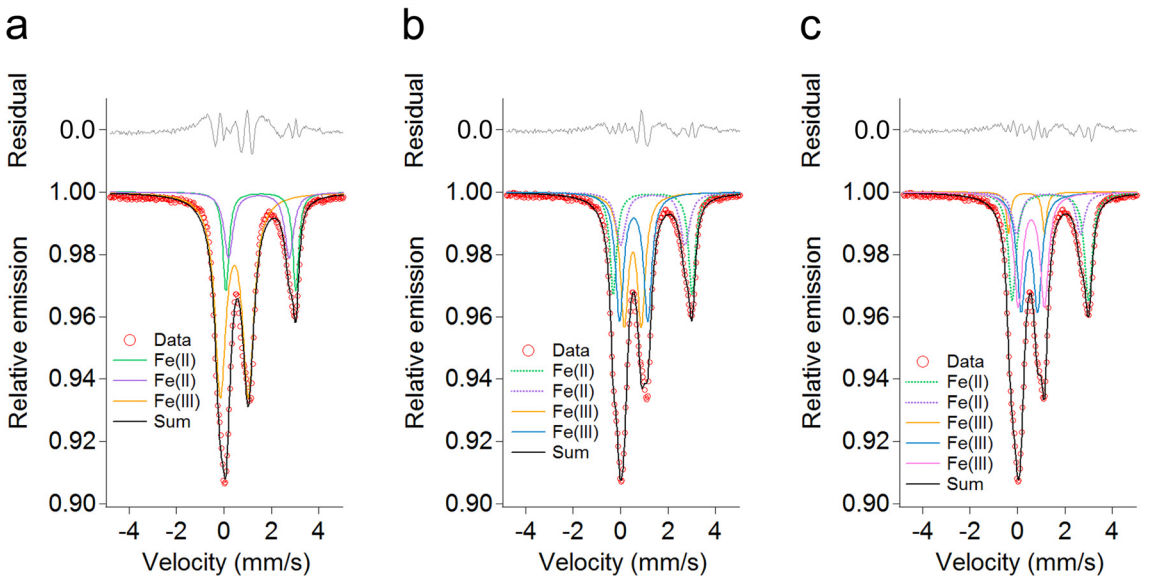
Supplementary Figure 2. Different models were used to simulate the Mössbauer spectrum of 50 ^{57}Fe per PfFtn 24-mer under anaerobic conditions (t = 0 s). (a) A model with one Fe(II) doublet (quadrupole doublet), (b) a model with two Fe(II) doublets, and (c) a model with three Fe(II) doublets. The residual (grey lines) shows the differences of the experimental data (red circles) and the superposition of the simulated subspectra (black line). Measurements were performed at 80 K.



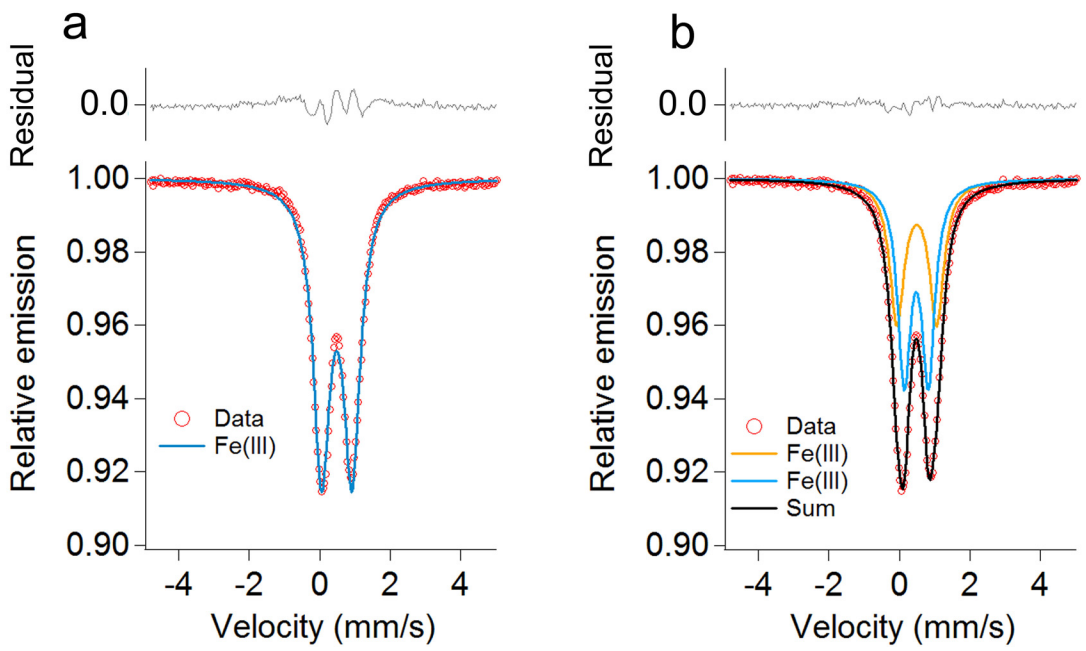
Supplementary Figure 3. Simulation of the Mössbauer spectrum of HuHF loaded with 50 Fe(II) per ferritin 24-mer anaerobically. (a) Simulation of the spectrum using a model of one Fe(III) and two Fe(II) doublets, and (b) using a model of one Fe(III) and three Fe(II) doublets. The Fe(III) is 'dirty' iron, which presumably has been formed due to oxidation of Fe(II) by molecular oxygen before addition to ferritin since Fe(II) was added under anaerobic conditions. The residual (grey lines) is the subtraction of experimental data (red circles) from the superposition of simulated subspectra (black line). Measurements were performed at 80 K.



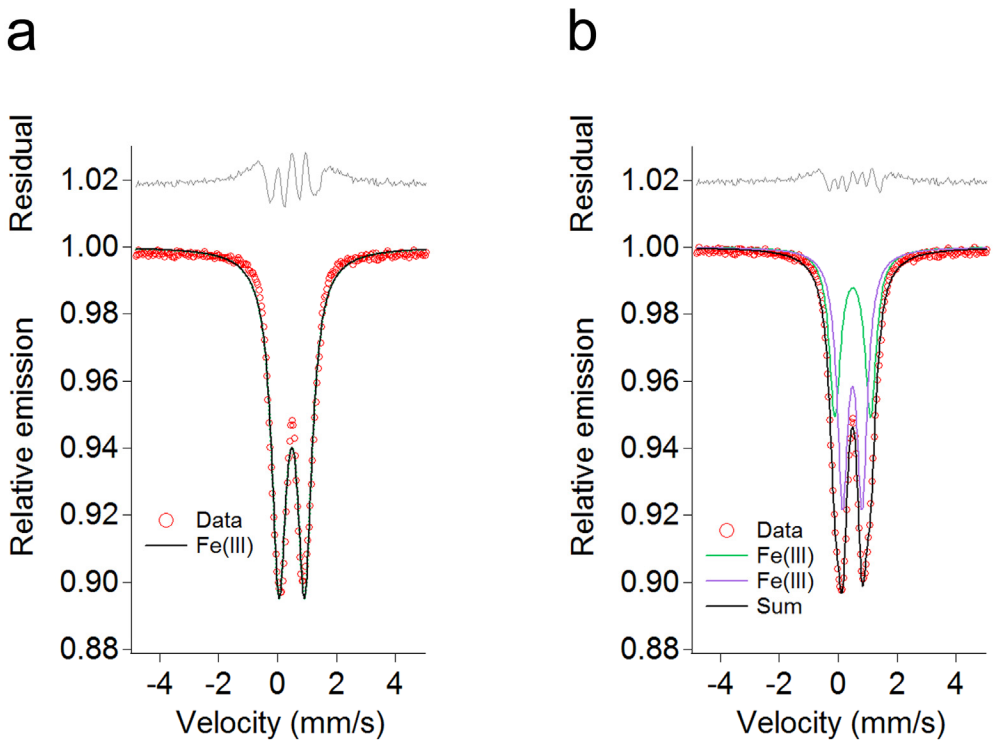
Supplementary Figure 4. Different models were used to simulate the Mössbauer spectrum of 50 ^{57}Fe per PfFtn 24-mer 0.7 s after addition of molecular oxygen. (a) A model with one Fe(II) and one Fe(III) doublets, and (b) a model with one Fe(II) and two Fe(III) doublets. The residual (grey lines) shows the differences of the experimental data (red circles) and the superposition of simulated subspectra (black line). Measurements were performed at 80 K.



Supplementary Figure 5. Simulation of the Mössbauer spectrum of HuHF loaded with 50 Fe(II) per ferritin 24-mer 0.7 s after reaction with dioxygen. (a) Simulation of the spectrum using a model of two Fe(II) and one Fe(III) doublets, (b) using a model of two Fe(II) and two Fe(III) doublets, and (c) using a model of two Fe(II) and three Fe(III) doublets. The residual (grey lines) is the subtraction of experimental data (red circles) from the superposition of simulated subspectra (black line). Measurements were performed at 80 K.



Supplementary Figure 6. Simulation of Mössbauer data of 50 ^{57}Fe per PfFtn 24-mer after complete oxidation of Fe(II) and formation of Fe(III) products. 300 s after addition of 50 ^{57}Fe per ferritin 24-mer under O_2 saturated condition the sample was frozen and Mössbauer data were recorded. (a) A model with one Fe(II) doublet, and (b) a model with two Fe(III) doublets. As can be seen a good fit to the Mössbauer data was obtained using a model with two Fe(III) doublets. The residual (grey lines) shows the differences of the experimental data (red circles) and the superposition of simulated subspectra (black line). Measurements were performed at 80 K.



Supplementary Figure 7. Simulation of the Mössbauer spectrum of HuHF loaded with 50 Fe(II) per ferritin 24-mer 60 s after reaction with dioxygen. (a) Simulation of the spectrum using a model of one Fe(III) doublet, and (b) using a model of two Fe(III) doublets. The residual (grey lines) is the subtraction of experimental data (red circles) from the superposition of simulated subspectra (black line). Measurements were performed at 80 K.

HuHF	...Glu27 (x6) Tyr34....	....Glu62 (x2) His65...	...Glu107...	...Gln141 (x2) Ala144...
PfFtn	...Glu17 (x6) Tyr24....	....Glu50 (x2) His53...	...Glu94...	...Gln127 (x2) Glu130...
BfMF	...Glu23 (x6) Tyr30....	....Glu58 (x2) His61...	...Glu103...	...Gln137 (x2) Asp140...
HMtF	...Glu27 (x6) Tyr34....	....Glu62 (x2) His65...	...Glu107...	...Gln141 (x2) Ser144...
PnFtn	...Glu15 (x6) Tyr22....	....Glu48 (x2) His51...	...Glu94...	...Gln127 (x2) Glu130...
EcFtnA	...Glu17 (x6) Tyr24....	....Glu50 (x2) His53...	...Glu94...	...Gln127 (x2) Glu130...
PaFtn	...Glu18 (x6) Tyr25....	....Glu51 (x2) His54...	...Glu93...	...Gln127 (x2) His130...

Supplementary Figure 8. An amino acid residue of site B varies among ferritins. Alignment of the amino aicd residues of sites A and B of the diiron ferroxidase center of various ferritins, shows that a residue of site B (Cyan) is different among ferritins. From top to bottom: human H-type ferritin (HuHF), *Pyrococcus furiosus* ferritin (PfFtn), bullfrog M ferritin (BfMF), human mitochondrial ferritin (HMtF), *Pseudonitzschia* ferritin (PnFtn), *Escherichia coli* ferritin A (EcFtnA), and *Pseudomonas aeruginosa* ferritin (PaFtn).

Supplementary Tables

Supplementary Table 1. Thermodynamic parameters of Fe(II) binding in sites A, B, and C of PfFnt and HuHF. These data were obtained using isothermal titration calorimetry.

Site		Thermodynamic parameters from reference [1]	
		Apo-PfFtn	Apo-HuHF
A	N	1	1.0 ± 0.1
	K (M^{-1})	(5.8 ± 1.3) × 10^5	(5.4 ± 0.8) × 10^5
	ΔH (kJ mol^{-1})	-16.6 ± 0.4	3.2 ± 0.2
B	N	1	0.9 ± 0.1
	K (M^{-1})	(5.5 ± 1.0) × 10^4	(1.5 ± 0.3) × 10^3
	ΔH (kJ mol^{-1})	11.3 ± 1.2	15.3 ± 0.3
C	N	1	0.9 ± 0.1
	K (M^{-1})	(1.0 ± 0.3) × 10^3	(1.5 ± 0.3) × 10^3
	ΔH (kJ mol^{-1})	18 ± 2.1	15.3 ± 0.3

Measurements with PfFtn were performed at 70 °C, which is close to physiological temperature of *P. furiosus*, and those with HuHF were performed at 25 °C. N is the stoichiometry of Fe(II) per subunit, K is the association constant, and ΔH is the enthalpy of binding.

Supplementary Table 2. The majority of Fe(III) intermediate species and Fe(III) products are spin coupled (EPR silent).

Sample	Time (s)	Mononuclear Fe(III) %	Fe(II)-Fe(III) %
HuHF	0	0.13	0
	0.7	2.1	0.2
	50	4.9	0
PfFtn	0	0.01	0.0
	0.7	1.2	0.1
	300	2.1	0.0

Data were obtained using EPR spectroscopy. Circa 2 Fe(II) per ferritin subunit was added. The numbers are the percentage of the spin concentration of each species. Concentration of HuHF was 55 μM (24-mer) and that of PfFtn was 45 μM (24-mer).

References.

1 Ebrahimi, K. H., Bill, E., Hagedoorn, P.-L. & Hagen, W. R. The catalytic center of ferritin regulates iron storage via Fe (II)-Fe (III) displacement. *Nature chemical biology* **8**, 941-948 (2012).

2 Bou-Abdallah, F. *et al.* Ferrous ion binding to recombinant human H-chain ferritin. An isothermal titration calorimetry study. *Biochemistry* **41**, 11184-11191 (2002).