Supplementary Information

Spectroscopic evidence for the presence of a high-valent Fe(IV) species in the ferroxidase reaction of ferritin

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Appendix S1

Materials and Methods.

Chemicals. All chemicals were reagent grade and were purchased from Sigma Aldrich.

Protein expression, purification, and preparation of apoferritin. *Pyrococcus furiosus* ferritin (PfFtn) was 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 (^{NAT}Fe(II)) 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 diluted sulfuric acid and was diluted in anaerobic Milli Q. water to reach the final 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 48 Fe(II) per ferritin 24-mer.

Stopped-flow UV-visible spectroscopy. A 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. 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 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. The final pH was 6.9. After mixing the Fe(II) and protein solutions, spectra were recorded from 300 nm to 740 nm. Final concentration of protein was 4.4 μM or 15 μM (24-mer).

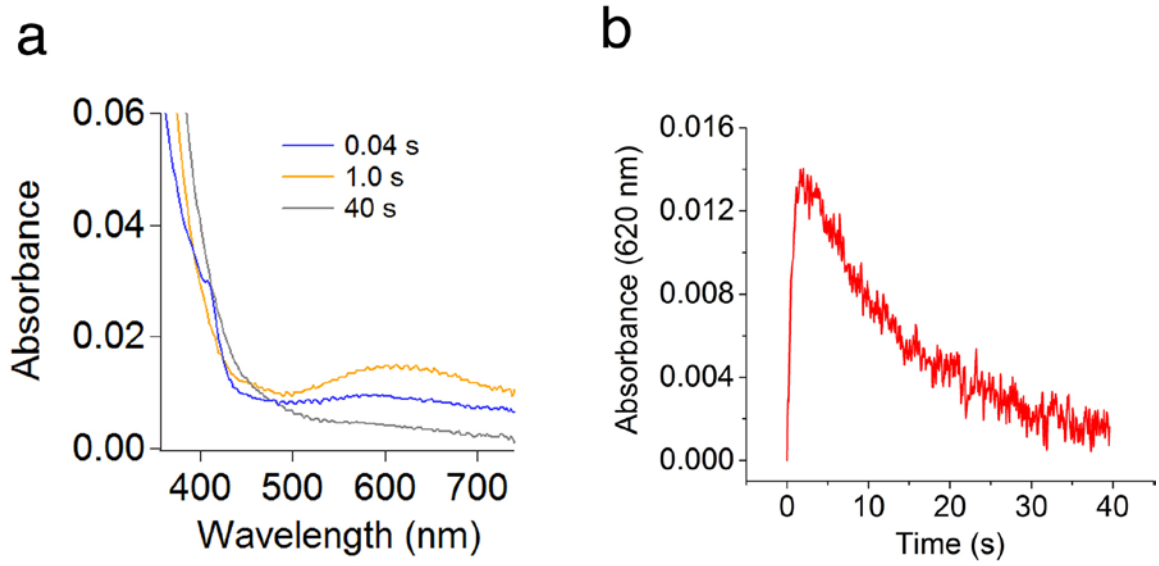
Freeze quench experiments. The reaction of PfFtn with 48 Fe(II) per ferritin 24-mer was quench as explained previously [3]. Final concentration of PfFtn was 45 μM (24-mer).

EPR spectroscopy. A Bruker ECS-106 EPR spectrometer was used to record the X-band EPR spectrums. EPR conditions were: modulation frequency 100 kHz; microwave power 0.127-201 mW; modulation amplitude, 12.7 or 4.02 Gauss; Temperature 6.4-30 K. Final concentration of PfFtn was 44 μM (24-mer).

Mössbauer spectroscopy. Mössbauer spectra were recorded as explained previously [3].

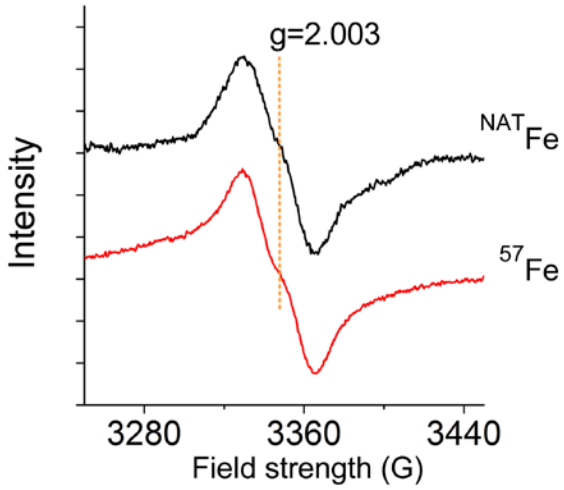
Singular value deconvolution (SVD) method. Experimental data as equidistant values of optical absorbance amplitudes versus time over a total period of 2-5 seconds were put in a rectangular matrix, which was then decomposed, according to the Singular Value Decomposition (SVD) method[4], into three matrices containing exclusively information on time courses, independent reaction components, and spectral properties, respectively. Since the presumed peroxodiferric intermediate is fully developed and metastable at the end of the measured time period, we used the simple model of two consecutive reactions with two independent rate constants (A → I¬1 → I2) to fit the SVD time matrix subject to a basis transformation, by systematic variation of the rate-constant values using a program written in LabVIEW. In this scheme A is the initial ferritin preparation, I1 is the presumed tyrosine radical intermediate, and I2 is the complex metastable intermediate whose spectrum is presumably composed of contributions from a peroxodiferric and an Fe(IV) species. The end result is an optimized set of rate constants plus the individual optical spectra of A, I1, and I2.

Supplementary Figures

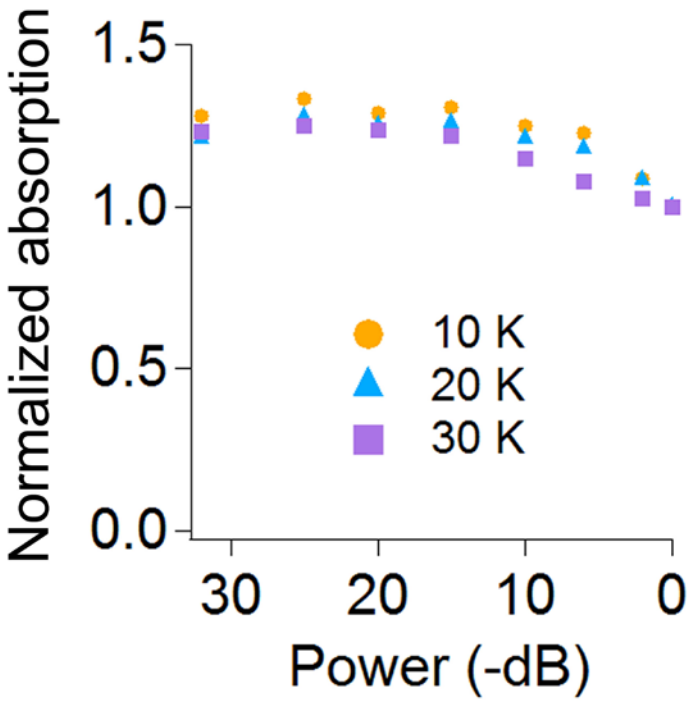


Supplementary Figure 1. UV-visible absorbance spectrum of Fe(II) oxidation by PfFtn. (a) UV-visible stopped-flow absorbance spectra were recorded for approximately 40 seconds after addition of 48 Fe(II) per ferritin 24-mer to PfFtn. (b) Progress curve of the formation and decay of the peroxodiferric intermediate at 620 nm for addition of 48 Fe(II) per ferritin 24-mer. Measurements were performed at 47 ºC.

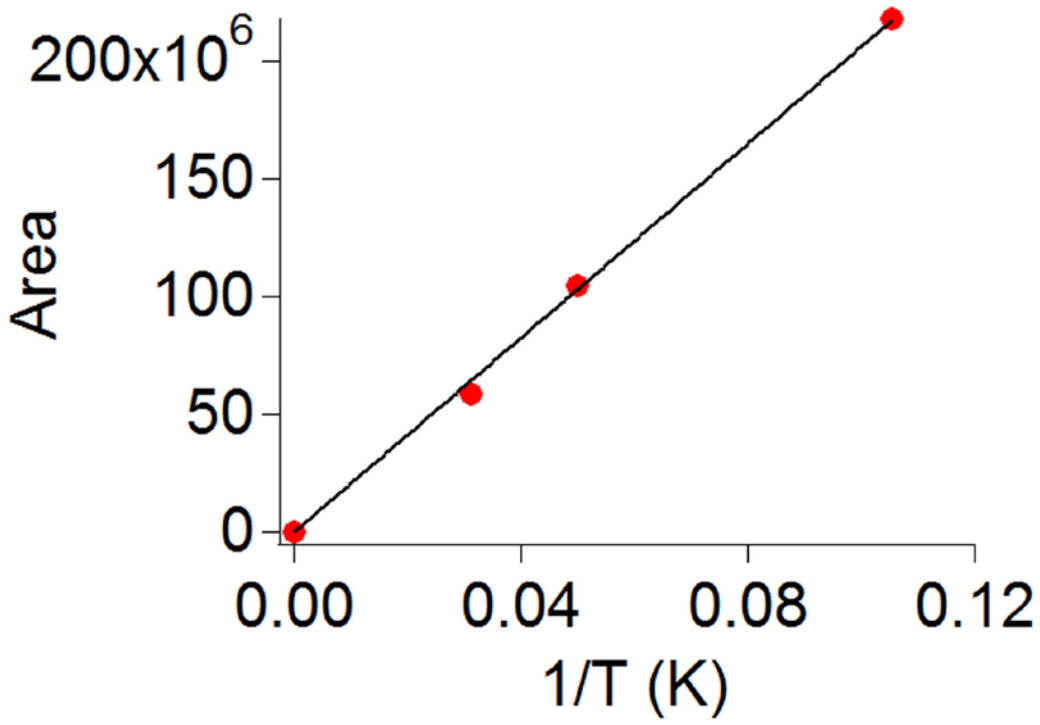
Figure 3



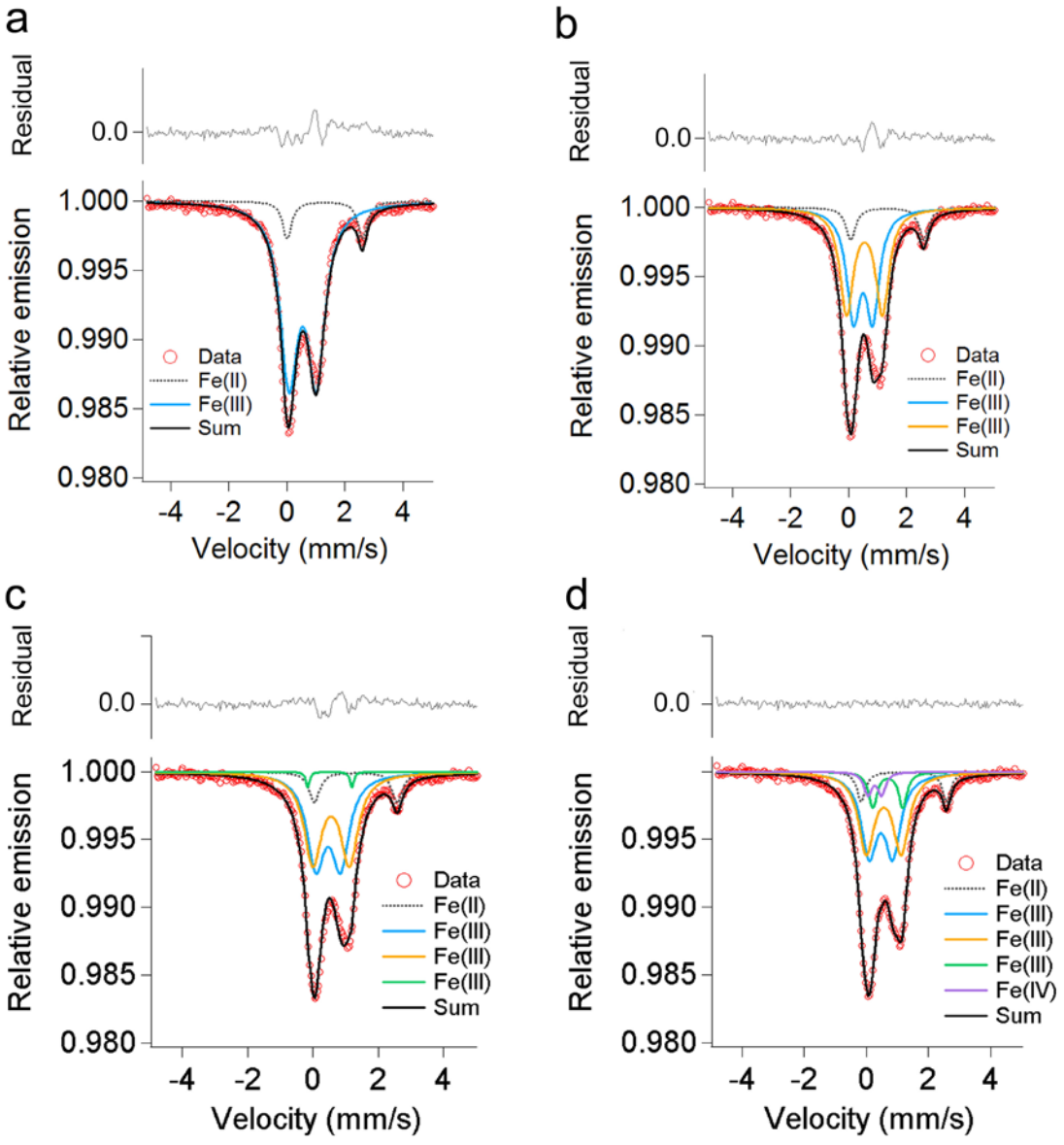
Supplementary Figure 2. EPR spectroscopy confirmed formation of a tyrosine radical near a metal center. The hyperfine effect of ^{57}Fe on the EPR spectrum at g=2.0031 in PfFtn. 48 ^{57}Fe(II) (red) or ^{NAT}Fe(II) (black) per ferritin 24-mer (44 μM) were added aerobically to PfFtn and the reaction was quenched after circa 1 s. Experimental parameters: microwave power 127 mW, modulation amplitude 4.02 gauss, temperature 7.0 K, microwave frequency 9.38791 GHz.



Supplementary Figure 3. A plot of the intensity of the g=2.0031 signal obtained using numerical double integration of the experimental derivative signal as a function of microwave power. The curve reveals very weak power saturation of the signal down to 10 K. EPR conditions: microwave frequency, 9.3917GHz; modulation frequency, 100 kHz; modulation amplitude, 12.7 Gauss. The intensities are normalized to the intensity of the signal at 0 dB power (201 mW). The absence of significant saturation even at very high microwave power (0 dB, or 201 mW) especially at 10 and 20 K is remarkable and would be not consistent with the behavior of an isolated organic radical.



Supplementary Figure 4. A plot of the double integral (area) of the g=2.0031 signal as a function of inverse temperature. EPR conditions: microwave frequency, 9.3891 GHz; microwave power 50.7 mW; modulation frequency, 100 kHz; modulation amplitude, 12.7 Gauss.



Supplementary Figure 5. Different models were used to simulate the Mössbauer spectrum of 48 ^{57}Fe per PfFtn 24-mer 1.0 s after addition of molecular oxygen. (a) A model with one Fe(II) and one Fe(III) doublets, (b) a model with one Fe(II) and two Fe(III) doublets, (c) a model with one Fe(II) and three Fe(III) doublets, and (d) a model with one Fe(II), three Fe(III), and one Fe(IV) doublets. The Mössbauer parameters of the Fe(II) and the two major Fe(III) doublets in (c and d) were from those obtained for the sample frozen 0.7 s after addition of dioxygen [3]. 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.

References

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2. Bou-Abdallah, F., Arosio, P., Santambrogio, P., Yang, X., Janus-Chandler, C. & Chasteen, N. D. (2002) Ferrous ion binding to recombinant human H-chain ferritin. An isothermal titration calorimetry study, *Biochemistry.* **41**, 11184-11191.
3. Ebrahimi, K. H., Bill, E., Hagedoorn, P.-L. & Hagen, W. R. (2016) Spectroscopic evidence for the role of a site of the di-iron catalytic center of ferritins in tuning the kinetics of Fe(ii) oxidation, *Molecular BioSystems.* **12**, 3576-3588.
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