Supporting Information

Spectroscopic Evidence for and Characterization of a Trinuclear Ferroxidase Center in Bacterial Ferritin from *Desulfovibrio vulgaris* Hildenborough[†]

Alice S. Pereira,*[,‡] Cristina G. Timóteo,[‡] Márcia Guiherme,[‡] Filipe Folgosa,[‡] Sunil G. Naik,[§] Américo G. Duarte,[‡] Boi Hanh Huynh,*[, §] and Pedro Tavares*[,‡]

[‡] *Requimte/CQFB, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Quinta da Torre, 2829-516 Caparica, Portugal, and* [§]*Department of Physics, Emory University, Atlanta, GA 30322, USA*

masp@fct.unl.pt, pabt@fct.unl.pt, vhuynh@emory.edu,

Construction of DvFtn overexpression vector

The overexpression vector was constructed using standard molecular biology protocols. All enzymes and competent cells used were from Invitrogen. *Desulfovibrio (D.) vulgaris* Hildenborough ATCC 29579 cells were grown anaerobically in lactate-sulfate Postgate C medium at 30 ºC (*S1*). Genomic DNA was isolated using a standard CTAB extraction method (*S2*). The full DvFtn encoding gene was amplified from *D. vulgaris* Hildenborough genomic DNA by PCR with two primers homologous to gene ends (5'-CCATGCTAGC**CATATG**CTCAGCCAGCGTATGAACG-3' and 5'-AGCTCCCGAG**GAATTC**CTACGCCGTCGTCGGCGG-3' from Sigma Genosys) containing a *Nde* I and *Eco*R I restriction sites (in boldface), respectively. The 510-bp PCR product was purified by agarose gel electrophoresis, hydrolyzed with *Nde* I and *Eco*R I, re-purified and ligated with T4 DNA polymerase to pET-21c (Novagen) previously digested with the same enzymes and gel purified, following the manufacturer instructions. The sequence of the coding gene was verified by automated DNA sequence (STAB Vida services, Portugal). The resulting expression vector was named pET-21c-DvFtn.

The E130A variant was prepared using the Quick Change Site-Directed Mutagenesis kit from Stratagene using pET-21c-DvFtn as template. The mutation was introduced by PCR with a mutagenic primer 5'-GACCGAACAGGTCGAG**GCC**GAGGATAGCGTCAACG-3' (GAA to GCC substitution in boldface) following the manufacturer's instructions. The sequence of the full coding gene was verified by DNA sequencing. The resulting plasmid was designated pET-21c-E130A-DvFtn.

Expression and Purification of wild-type DvFtn and E130A variant

E. coli BL21(DE3) competent cells transformed with wild-type encoding expression vector pET-21c-DvFtn or with pET-21c-E130A-DvFtn were grown in 10 mL of LB medium containing 0,1 g/L of ampicillin, at 37 ºC and 250 rpm for 7-8 h. This culture (1 mL) was used to inoculate 100 mL of defined M9 minimum medium, free of iron compounds, containing 0.1 g/L of ampicillin. The culture was grown overnight at 37 ºC, and 250 rpm. 0,5 L of M9 minimum medium with 0.1 g/L ampicillin were then inoculated with 10 mL of the previous pre-culture and let grow, at 37 ºC, until an $OD_{600\ nm}$ of approximately 2,0 was reached. Expression of recombinant proteins was induced by addition of 0.05

mM of IPTG (isopropyl-β-D-1-thiogalactopyranoside). At this moment, 0.5 L of fresh M9/ampicillin medium was added. Overnight grown cultures were used to prepare the crude extracts. Cells were harvested by centrifugation at 5,700*g* for 15 min. Typically a yield of approximately 4 g of wet cell paste/L of culture was obtained.

The cell pellet from 1 L-culture was re-suspended in cold 10 mM Tris-HCl, pH 7.6 buffer. Cell lysis was performed in a French press at 1,700 Psi. To avoid proteolysis 10 mM benzamidine hydrochloride and 1 mM PMSF (phenylmethanesulfonyl fluoride) were added to the lysed suspension. The crude extract was cleared from cell debris and membrane fraction by low-speed (5,700*g*) centrifugation followed by ultracentrifugation (138,000*g*) for 1 h at 4 ^{o}C. The supernatant was then dialyzed overnight at 4 ^{o}C against 10 mM Tris-HCl pH 7.6 for protein purification.

Recombinant wild-type and E130A DvFtn proteins were purified in a single chromatographic step using a DEAE-Sepharose Fast Flow column from GE Healthcare (2.6×30 cm) equilibrated with 10 mM Tris-HCl, pH 7.6 (buffer A). Elution of adsorbed proteins was achieved with a linear gradient of 150 – 300 mM NaCl in buffer A. Fractions containing the recombinant proteins were pooled and concentrated in an ultrafiltration cell (Vivacell 70 with a 30 kDa membrane from Sartorius). The protein was dialyzed against 0.2 M Tris-HCl, pH 7.6, 0.2 M NaCl buffer. Protein purity was assessed by SDS-PAGE. A typical purification yield was 80 mg of pure protein/L of cell culture.

Mössbauer Spectral Analysis

The Mössbauer spectra were analyzed by using a spin Hamiltonian ($\boldsymbol{H}_S$) formulism containing both the electronic ($\boldsymbol{H}_e$) and hyperfine ($\boldsymbol{H}_{hf}$) interactions.

$$\boldsymbol{H}_S = \boldsymbol{H}_e + \boldsymbol{H}_{hf} \quad \text{(S1)}$$

For the mixed valence $Fe^{2+}Fe^{3+}$ species, a strong antiferromagnetic interaction between the two Fe ions is assumed. The electronic energy levels of the $S = 1/2$ ground state is described by,

$$\boldsymbol{H}_e = \beta \boldsymbol{S} \cdot \boldsymbol{g} \cdot \boldsymbol{H} \quad \text{(S2)}$$

and the hyperfine interactions by,

$$\boldsymbol{H}_{hf} = \sum_{i=1}^{2} \frac{eQ(V_{zz})_i}{4} \left[\boldsymbol{I}_{zi}^2 - \frac{I_i(I_i+1)}{3} + \frac{\eta}{3}\left(\boldsymbol{I}_{xi}^2 - \boldsymbol{I}_{yi}^2\right) \right] + \sum_{i=1}^{2} (\boldsymbol{S} \cdot \boldsymbol{A}_i \cdot \boldsymbol{I}_i - g_n \beta_n \boldsymbol{H} \cdot \boldsymbol{I}) \qquad \text{(S3)}$$

For the diamagnetic diferrous center, the same spin Hamiltonian with $S = 0$ is applied. Thus, only the first and last terms of equation (S3) are required.

In simulating the spectrum of mononuclear Fe^{2+} species shown in Figure 7, we assume that $S = 2$, and that

$$\boldsymbol{H}_e = D\left[\boldsymbol{S}_z^2 - \frac{S(S+1)}{3} + \frac{E}{D}\left(\boldsymbol{S}_x^2 - \boldsymbol{S}_y^2\right)\right] + \beta \boldsymbol{S} \cdot \boldsymbol{g} \cdot \boldsymbol{H} \qquad \text{(S4)}$$

and

$$\boldsymbol{H}_{hf} = \frac{eQV_{zz}}{4} \left[\boldsymbol{I}_z^2 - \frac{I(I+1)}{3} + \frac{\eta}{3}\left(\boldsymbol{I}_x^2 - \boldsymbol{I}_y^2\right) \right] + (\boldsymbol{S} \cdot \boldsymbol{A} \cdot \boldsymbol{I} - g_n \beta_n \boldsymbol{H} \cdot \boldsymbol{I}) \qquad \text{(S5)}$$

The parameters used for the simulation are listed in the text.

References

(S1) Postgate, J. R. 1984. The sulphate-reducing bacteria, 2nd ed., p. 32-33. Cambridge University Press, Cambridge.

(S2) Reichardt, M., and Rogers, S. (1998). Preparation and analysis of DNA. *In* Current Protocols in Molecular Biology, F.M. Ausubel, R. Brent, and R.E. Kingston, eds. (New York: John Wiley & Sons).

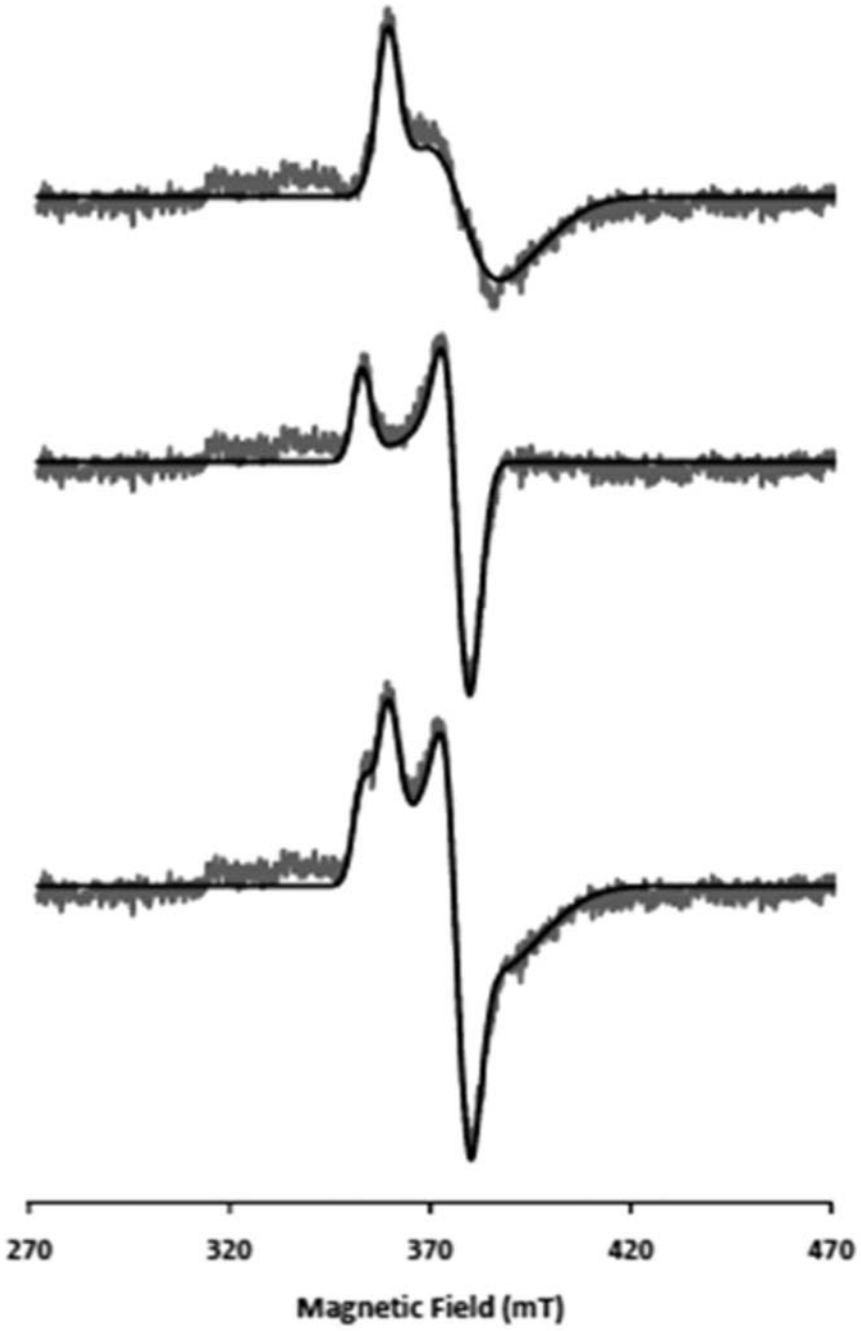

Figure S1. Decomposition of the EPR spectrum of Fe^{57}-enriched DvFtn loaded with 16Fe/24-mer (bottom spectrum, which is also shown in Figure 3). The spectrum were analyzed as a superposition of two overlapping $S = 1/2$ spectra (middle and top spectra). The middle spectrum accounted for (58 ± 5)% of the EPR signal while the top spectrum accounted for (42 ± 5)%. The solid line overlaid with the middle spectrum was simulated with principle g values of 1.96, 1.84 and 1.82 using line widths of 4.5 mT, 5.5 mT and 5.5 mT, respectively. The solid line overlaid with the top spectrum was simulated with principle g values of 1.92, 1.82 and 1.75 using line widths of 5.0 mT, 13 mT and 20 mT, respectively. The solid line overlaid with the bottom spectrum is the composite spectrum.

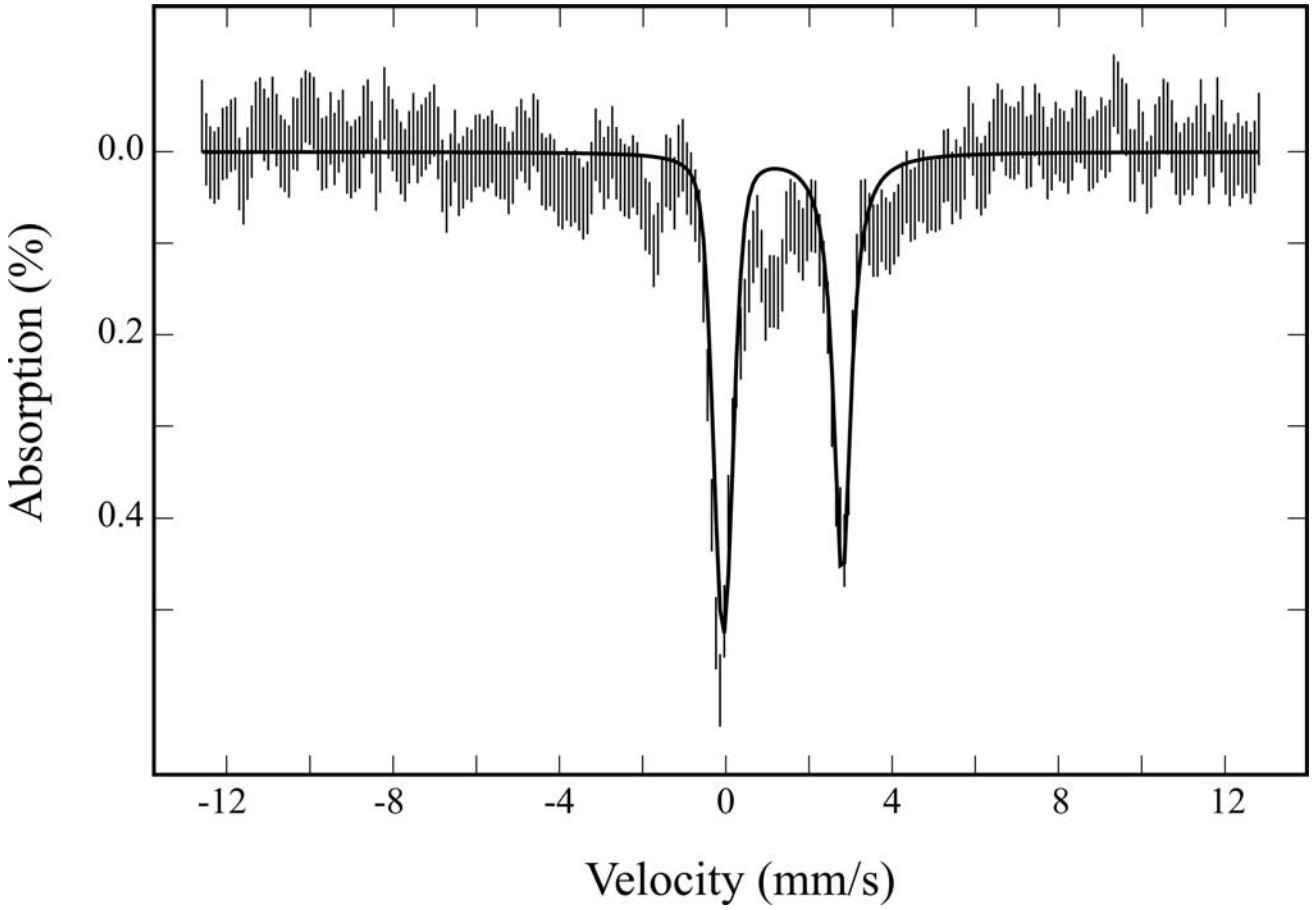

Figure S2. Mössbauer spectrum of the mononuclear Fe^{2+} species at 4.2 K and in a parallel field of 50 mT. This spectrum was prepared from the raw experimental data using the following procedure. Detailed analysis of the Mössbauer data indicates that the spectrum shown in Figure 6B contains 45% diamagnetic diferrous and 10% mononuclear Fe^{2+} absorption contributions, while spectrum 6C contains 20% diamagnetic diferrous and 20% mononuclear Fe^{2+} contributions. Thus, subtracting 44% of spectrum 6B from spectrum 6C removes completely the 20% diamagnetic diferrous contributions in spectrum 6C and reveals the quadrupole doublet (15.6%) arising from the mononuclear Fe^{2+}. The solid line is a theoretical simulation using the parameters list in Table 1 for the mononuclear Fe^{2+} species. The broad absorptions detected on the wings of the doublet are the remnant absorption (40%) of the mixed valence $Fe^{2+}Fe^{3+}$ center.