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Evidence for the synthesis of an unusual high spin (S=7/2) [Cu-3Fe-4S] cluster in the radical-SAM enzyme RSAD2 (viperin)

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Material and Methods

Chemicals. All chemical were reagent grade and were purchased from Sigma Aldrich or Fulka.

Expression, purification, and activation of protein. The expression and purification of fungal RSAD2 (viperin) was performed as explained previously [1]. Concentration of protein was determined using Bradford assay.

Preparation of [Cu-3Fe-4S] cluster. 1 ml of as isolated protein (Typically 100-150 uM) was incubated under strict anaerobic conditions (O2 < 5 ppm) with ferricyanide in a glove box (Belle Technology) over night. 20 µl ferricyanide was added to a final concentration of 2 mM. Subsequently, ferricyanide was removed using PD-10 desalting column. The column was equilibrated with 200 mM Tris, 300 mM NaCl, pH 7.5 containing 10 mM DTT. Subsequently, $CuSO_4$ solution (12.5 mM) prepared anaerobically using Milli-Q water was added. $CuSO_4$ solution was added in 10 steps each step 5 µl. Then, the solution was incubated under anaerobic conditions for at least 2 hours with gentle mixing. The excess copper and some aggregated protein were removed using PD-10 desalting column. The flow through the PD-10 column was collected and concentrated to prepare the samples for electron paramagnetic resonance (EPR) spectroscopy or resonance Raman (RR) spectroscopy.
To test if [4Fe-4S] cluster could be recovered after treatment of the enzyme with copper, Fe(II) was added to the enzyme containing copper reconstituted cluster under reducing conditions. After 10 minutes incubation in the glove box, the sample was directly used for further characterization using EPR spectroscopy.

UV-visible spectroscopy. To measure the UV-visible absorbance spectra, 10 µl of each concentrated sample prepared for resonance Raman spectroscopy was diluted in 990 µl of Tris buffer (100 mM) containing 300 mM NaCl (pH 7.6) in a glove box. The mixture was added to a quarts cuvette and tightly closed. Then the sample was transferred outside the glove box and the spectrum was recorded immediately using a UV-visible spectrometer. Measurements were done at room temperature.

Preparation of samples for EPR and RR spectroscopies. Native RSAD2 containing [4Fe-4S] or the enzyme containing [Cu-3Fe-4S] cluster was reduced or oxidized under strict anaerobic conditions in a glove box by addition of 5 equivalent (to enzyme concentration) of sodium dithionite o ferricyanide respectively. Subsequently, for EPR spectroscopy 200 µl of each sample was added to an EPR tube and the tube was tightly closed. The, the sample was transferred outside the glove box and frozen in liquid nitrogen immediately. To prepare samples for resonance Raman spectroscopy (~10 µl of approximately 1 mM enzyme) of the reduced or oxidized enzyme was added to an Eppendorf tube in the glove box. The tube was tightly closed and transferred outside and immediately frozen using liquid nitrogen. Subsequently, the samples were transferred on dry ice to the RR facility and stored in liquid nitrogen. Before measuring

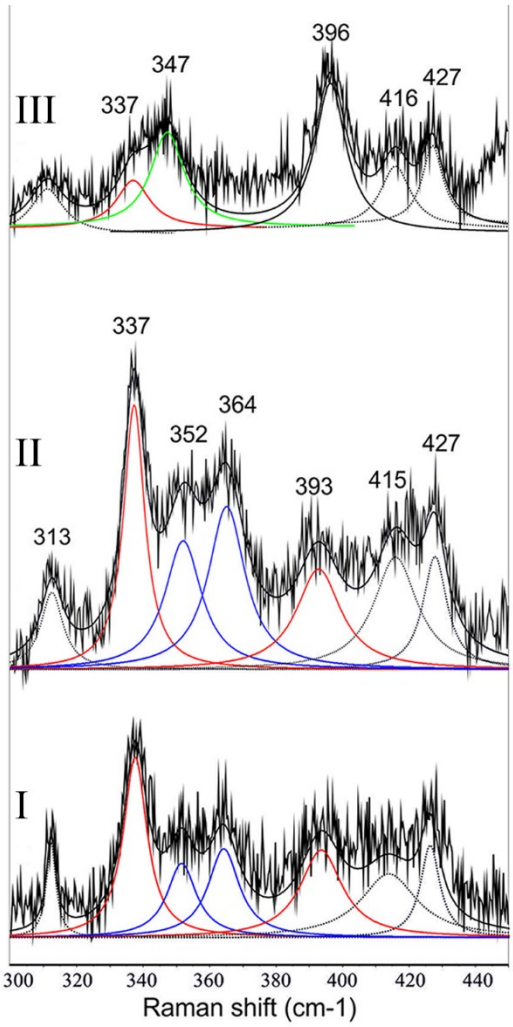
the RR spectra, each sample was thawed under nitrogen-5 flow and a 2 µl aliquot of sample was transferred to a liquid nitrogen cooled RR sample holder.

Electron paramagnetic resonance (EPR) spectroscopy. A Bruker EMX spectrometer equipped with an X-band super-high-sensitivity probehead (Bruker Biospin) and a low temperature helium flow cryostat was used to measure CW X-band EPR spectra. The EPR spectrum of buffer (Tris 100 mM, NaCl 300 mM pH 7.6) was used as background to correct the EPR spectrum of each sample. Programs demonstrated by Hagen, W. R. [2] were used to analyze the EPR spectra. EPR measurements were performed at different temperatures (4-70 K). EPR conditions were: gain 50 dB, modulation amplitude 10 gauss, microwave power 2.0 mW. Microwave frequency was between 9.3 and 9.4 GHz. Concentration of the EPR spectrum of the reduced $[4Fe\text{-}4S]^{1+}$ cluster was determined as explained previously [1] using a Cu(II)-EDTA standard.

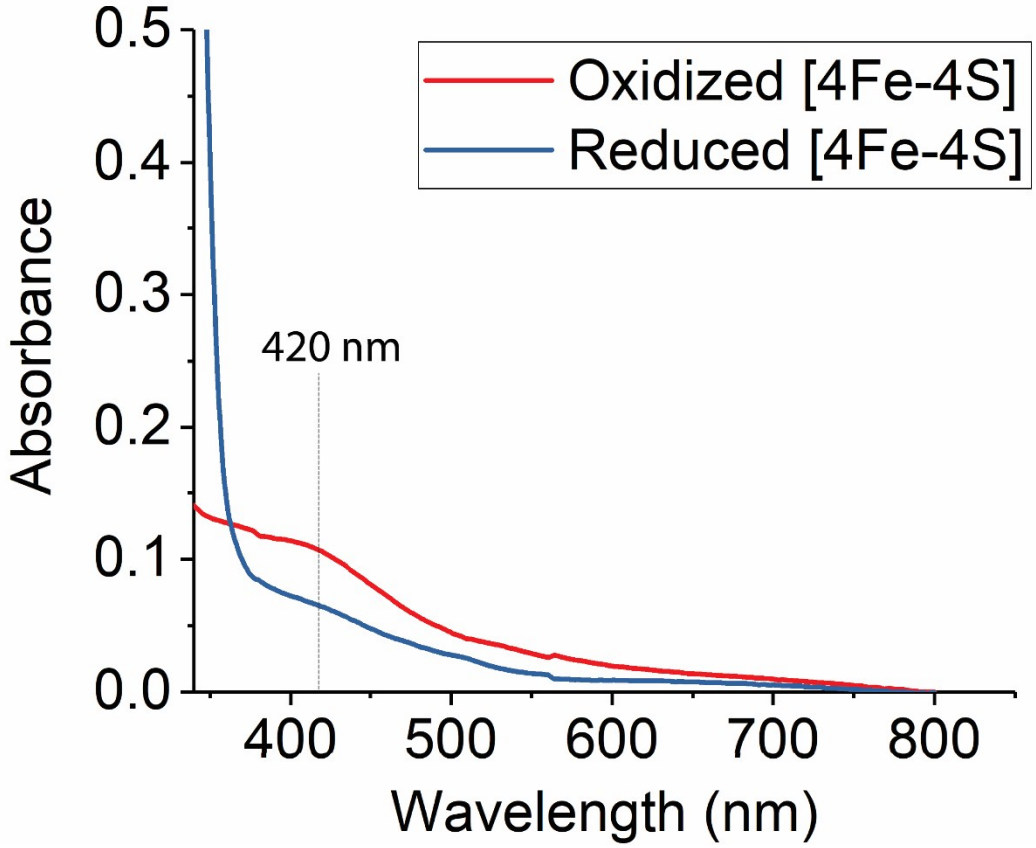
Resonance Raman (RR) spectroscopy. For RR spectroscopic experiments, about 2 µL of approximately 1 mM protein (in 100 mM Tris-HCl, pH 7.6, 300 mM NaCl) was introduced into a liquid-nitrogen-cooled cryostat (Linkam), mounted on a microscope stage and cooled down to 77 K. Spectra from the frozen sample were collected in backscattering geometry using a confocal microscope coupled to a Raman spectrometer (Jobin Yvon U1000) equipped with 1200 1/mm grating and a liquid-nitrogen-cooled CCD detector. The 413 nm line from a krypton ion laser (Coherent Innova 302) was used as excitation source. Typically, spectra were accumulated for 120 s with a laser power of 3–7 mW at the sample. The background scattering was removed by subtraction of a polynomial function and the spectra were deconvlouted using home-made software.

Ferene assay. To measure the amount of Fe(III) in protein ferene assay was used as demonstrated previously [1, 3]. First using ferrous ammonium sulfate a standard curve was obtained. For the assay 100 µl of solution containing different amounts of ferrous ammonium sulfate (10 to 200 µM) was mixed with 100 µl HCL (1% w/v). The solution was incubated at 100 C for 10 minutes. Subsequently, 500 µL Ammonium acetate (15% W/v), 100 µl ascorbic acid (4% W/v), 100 µl SDS (2.5% w/v), and 100 µl ferene (1.5% w/v) were added in order. The solutions were subjected to centrifugation at 9000 rpm for 10 min. Then the absorbance was measured at 593 nm. For the protein samples the same steps were followed. 100 ul of protein sample containing 9-20 µM protein was used.

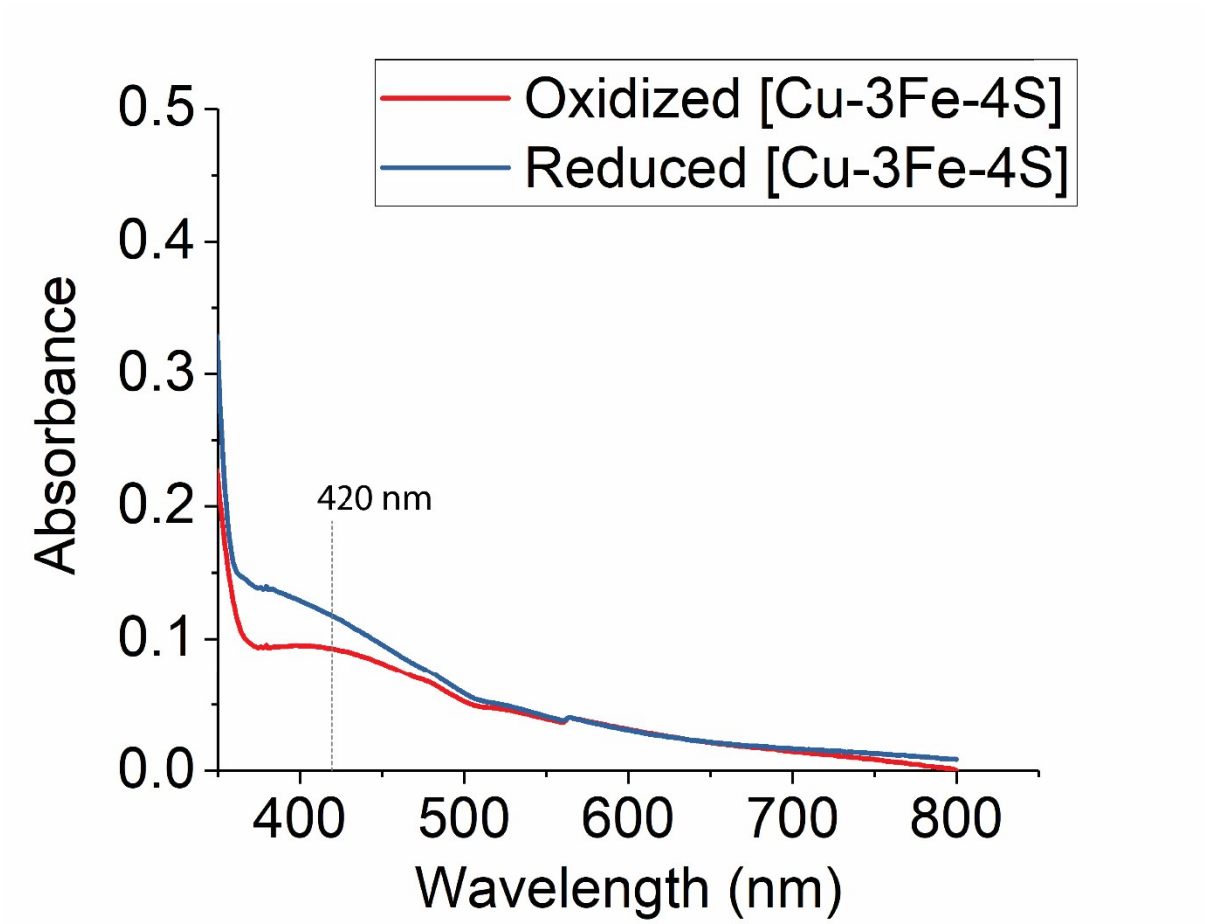
Supplementary Figures



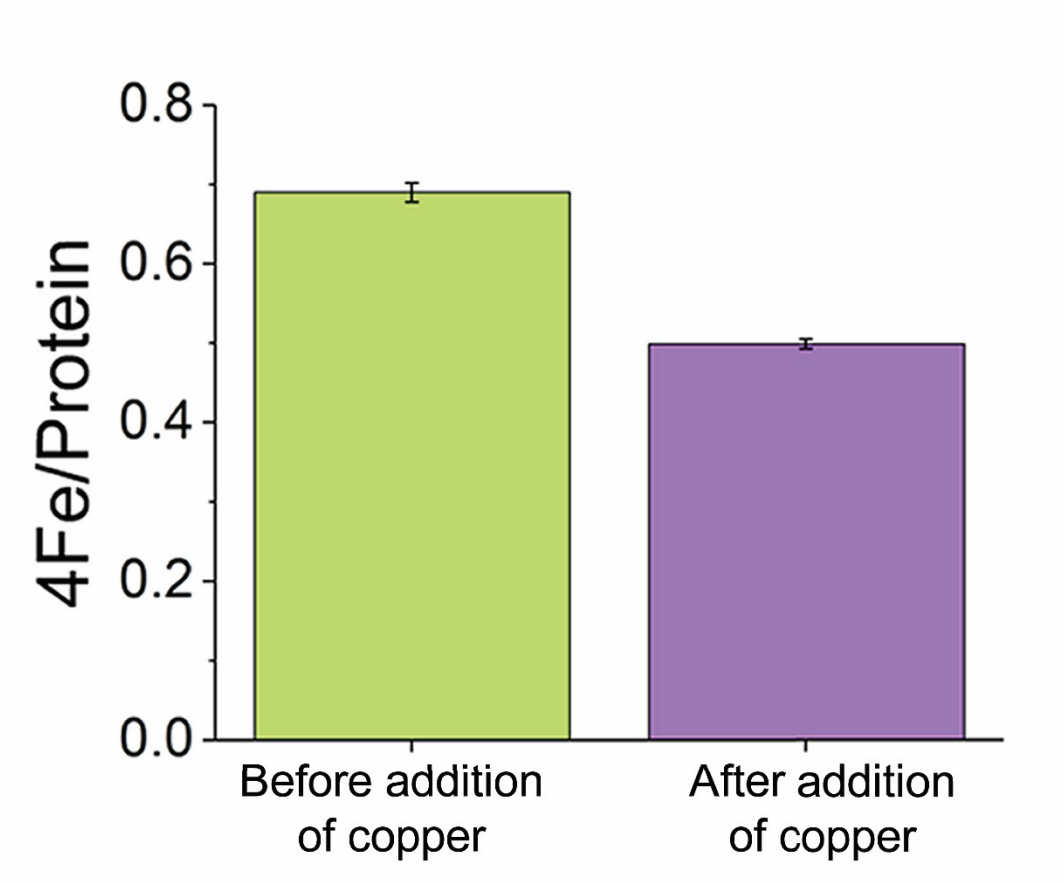
Supplementary Figure 1. Characterization of the [4Fe-4S] and [Cu-3Fe-4S] cluster using resonance Raman spectroscopy. (I) Oxidized native enzyme, (II) reduced native enzyme, (III) reduced and air exposed enzyme. Measurements were performed at 77 K using 413 nm excitation and 3-7 mW laser power.



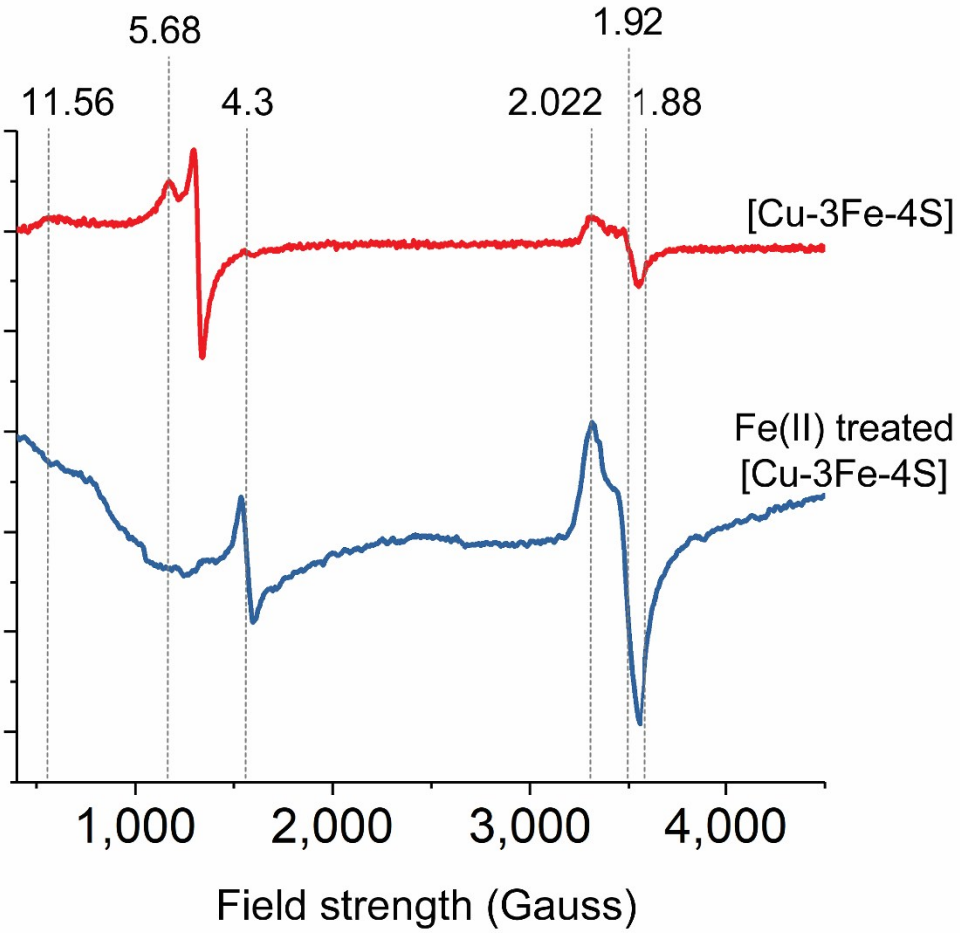
Supplementary Figure 2. UV-visible absorbance spectrum of the [4Fe-4S] cluster. The spectra were recorded under reducing conditions by addition of sodium dithionite (5 equivalent of enzyme) or under oxidizing conditions by addition of ferricyanide (5 equivalent of enzyme). A peak at 420 nm characteristics of the $[4Fe\text{-}4S]^{2+}$ cluster is observed. Measurements were performed at room temperature.



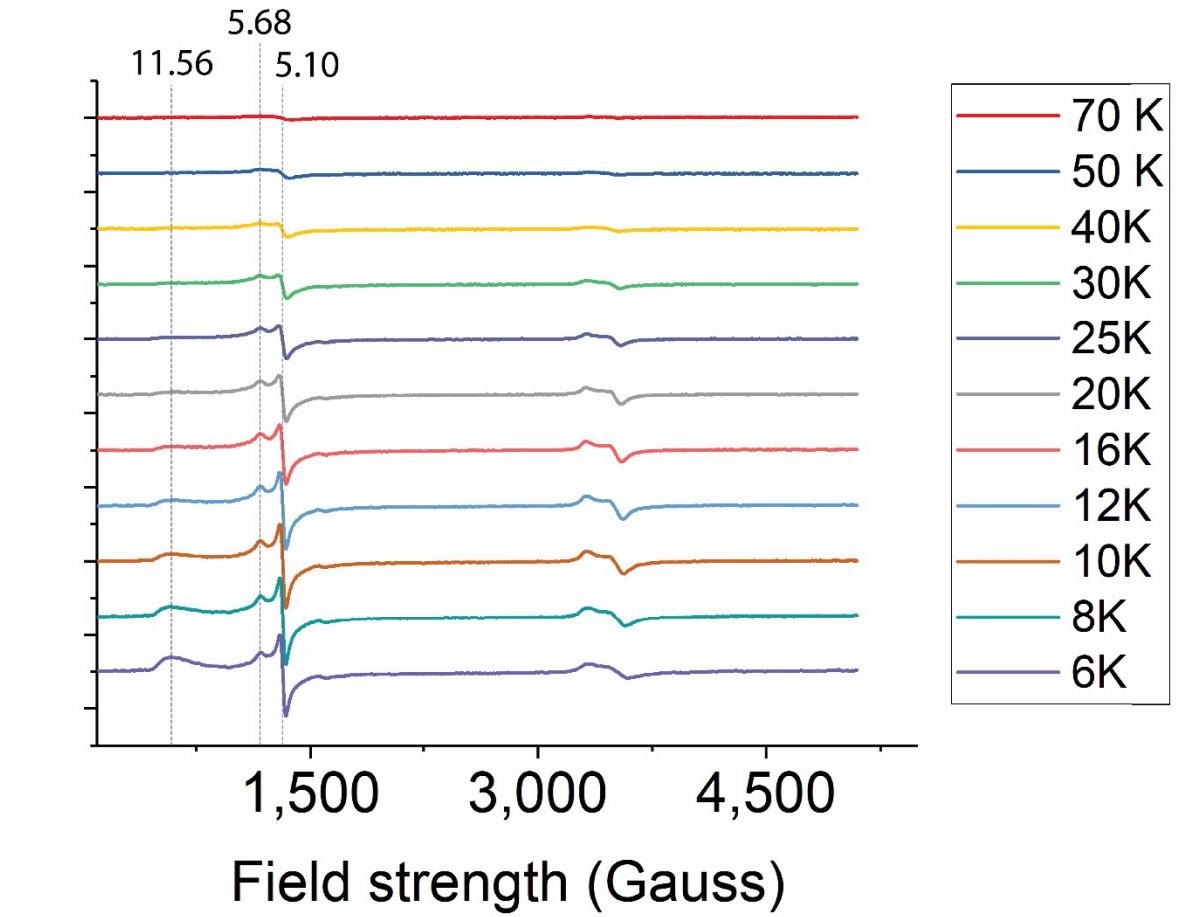
Supplementary Figure 3. UV-visible absorbance spectrum of RSAD2 after treatment with copper. The spectra were recorded under reducing conditions by addition of sodium dithionite (5 equivalent of enzyme) or under oxidizing conditions by addition of ferricyanide (5 equivalent of enzyme). The reduced enzyme did not show the typical absorbance peak at 420 nm characteristics of the $[4Fe\text{-}4S]^{2+}$ cluster (Supplementary Fig. 2). Moreover, the reduced enzyme has a higher absorbance at 420 nm compared to the oxidized enzyme. This is opposite of what is observed for the enzyme before treatment with copper, which contains [4Fe-4S] cluster (Supplementary Fig. 2). Measurements were performed at room temperature.



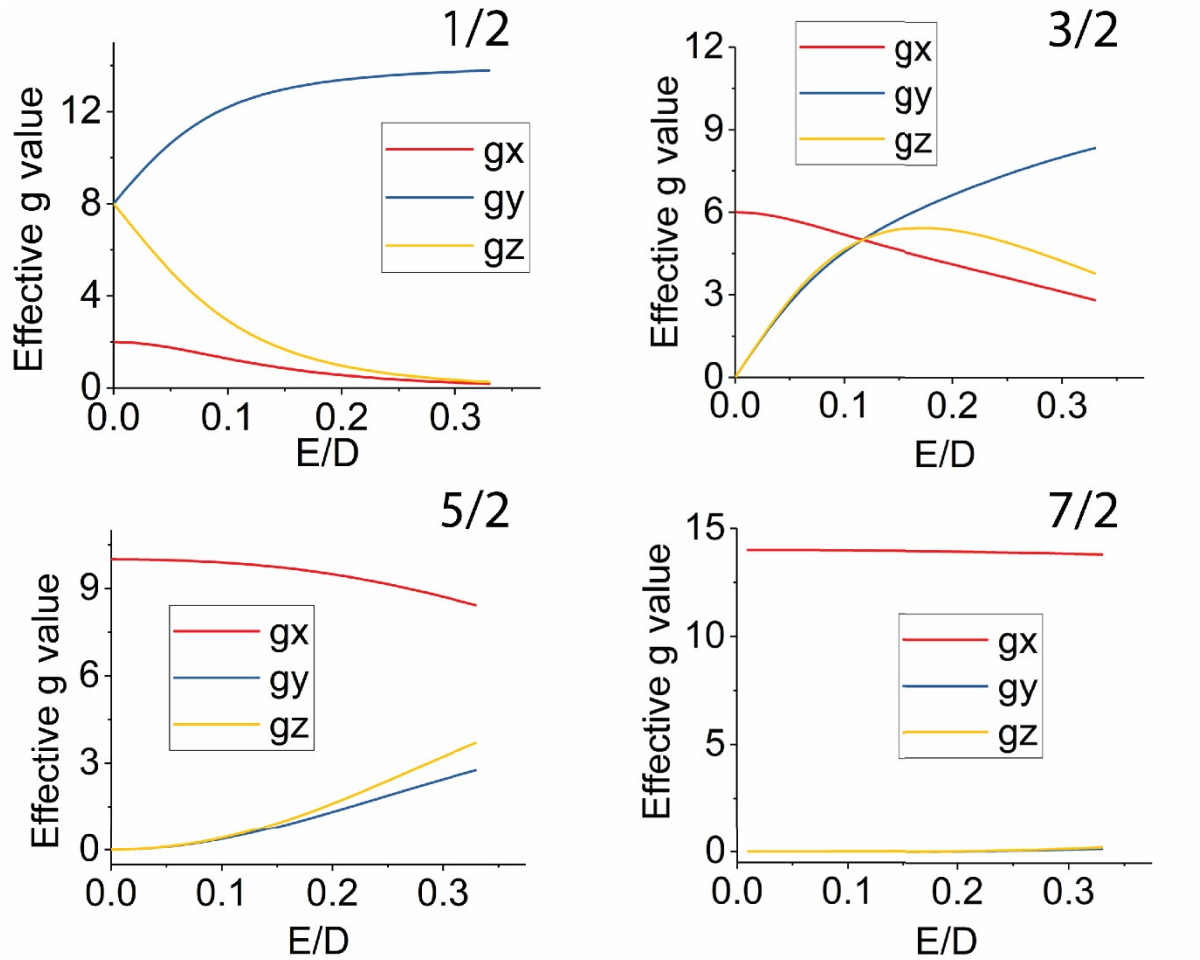
Supplementary Figure 4. The amount of iron measure in native enzyme containing [4Fe-4S] cluster before addition of copper (green) and the enzyme treated with copper (purple). Treatment of RSAD2 with copper under reducing conditions led to the removal of approximately one iron ion from the [4Fe-4S] cluster. Data are corrected for concentration of protein and are average of three measurements. Concentration of enzyme in each sample before and after treatment with copper was slightly different. Concentration of enzyme was approximately 90 μM for samples before addition of copper and 120 μM for samples after addition of copper.



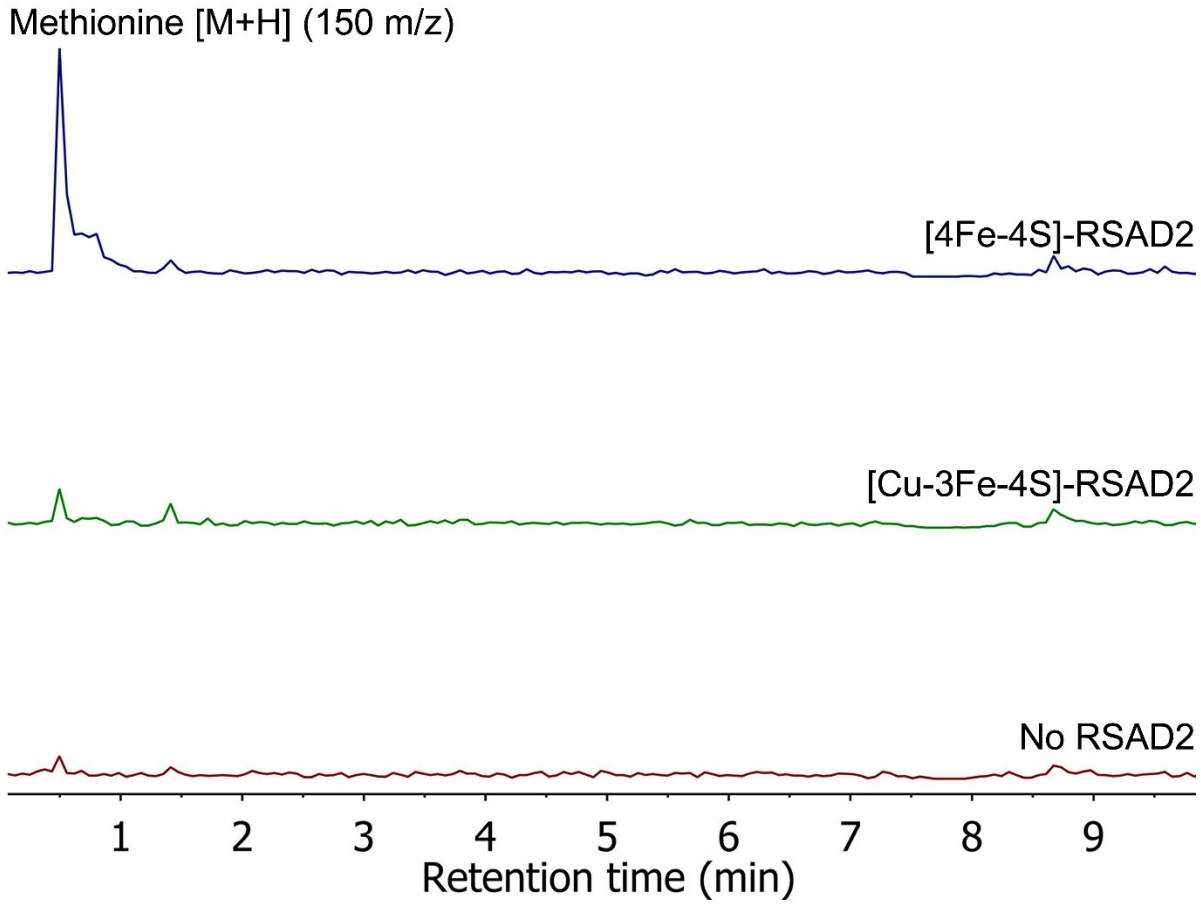
Supplementary Figure 5. EPR spectra of [Cu-3Fe-4S] cluster and the recovered $[4Fe\text{-}4S]^{1+}$ cluster. EPR spectra were recorded for enzyme containing [Cu-3Fe-4S] cluster (top trace), and after addition of Fe(II) (5-fold more than the protein concertation) to copper reconstituted enzyme under reducing conditions (bottom trace). Upon addition of Fe(II) the EPR lines assigned to the $[4Fe\text{-}4S]^{1+}$ cluster were recovered. The spectra were measured at 15 K under non-saturating conditions.



Supplementary Figure 6. Temperature dependence of the EPR lines of the EPR spectrum of the reduced [Cu-3Fe-4S] cluster under non-saturating conditions (Supplementary methods).



Supplementary Figure 7. Rhombograms of a system with S=7/2. The rhombograms are obtained for different Kramer's doublets. The rhombograms are obtained based on the diagonalization of the energy matrix method as explained by Hagen[2].



Supplementary Figure 8. The synthetic [Cu-3Fe-4S] cluster in the radical-SAM enzyme RSAD2 is not able to reductively cleave SAM. Formation of methionine [M+H] (m/z 150) was recorded as a measure of the reductive cleavage of SAM.

References.

1. K. H. Ebrahimi, S. B. Carr, J. McCullagh, J. Wickens, N. H. Rees, J. Cantley and F. A. Armstrong, The radical-SAM enzyme Viperin catalyzes reductive addition of a 5′-deoxyadenosyl radical to UDP-glucose in vitro, *FEBS Letters*, 2017, **591**, 2394-2405.
2. W. R. Hagen, *Biomolecular EPR spectroscopy*, CRC press, 2008.
3. K. Honarmand Ebrahimi, E. Bill, P.-L. Hagedoorn and W. R. Hagen, The catalytic center of ferritin regulates iron storage via Fe(II)-Fe(III) displacement, *Nature Chemical Biology*, 2012, **8**, 941.