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Generation of High-Spin Iron(I) in a Protein Environment Using Cryoreduction

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Supporting Information

ABSTRACT: High-spin Fe^{1+} sites are potentially important in iron−sulfur proteins but are rare in synthetic compounds and unknown in metalloproteins. Here, we demonstrate a spectroscopically characterized example of high-spin non-heme Fe^{1+} in a protein environment. Cryoreduction of Fe^{2+}-substituted azurin at 77 K with ^{60}Co γ radiation generates a new species with a $S = {}^3/_2$ (high-spin) Fe^{1+} center having $D > 0$ and $E/D \sim 0.25$. This transient species is stable in a glycerol−water glass only up to ∼170 K. A combination of electron paramagnetic resonance and Mössbauer spectroscopies provides a powerful means of identifying a transient high-spin Fe^{1+} site in a protein scaffold.

Many redox reactions in biology use cofactors with Fe−S bonds. For example, FeS clusters use iron (in a high-spin electronic configuration) for transferring electrons at low potential[1] and for reduction of challenging substrates like N_2.[2] Highly reduced FeS clusters include all-Fe^{2+} FeS clusters and synthetic analogues.[3] The next reduction step beyond all-Fe^{2+} would generate subferrous centers with high-spin Fe^{1+}. The generation of super-reduced FeS intermediates may underlie the binding and activation of N_2 by FeMoco, the active site of nitrogenase.[4] Few high-spin Fe^{1+} complexes are known,[5] with only two examples of synthetic high-spin Fe^{1+} complexes supported by S donors.[6]

Low-spin Fe^{1+} sites, on the other hand, have been characterized in detail, particularly in the Fe-only hydrogenase enzymes.[7] Because the understanding of high-spin Fe^{1+} has lagged behind that of low-spin Fe^{1+}, it remains a challenge to discover the key identifying characteristics of high-spin Fe^{1+}, which has never been observed in a protein environment.

Radiolytic reduction of metalloproteins at low temperatures (cryoreduction) is an effective way to generate highly reduced species, which may retain catalytic activity.[8] Cryoreduction has been used most commonly to activate oxyferrous monooxygenases but has also been shown to reduce some hemoproteins from Fe^{2+} to formally Fe^{1+} states.[9] Radiolytic and chemical reduction have also been used to reduce synthetic phthalocyanine−Fe^{2+} complexes.[10] In the previous cases where reduction occurs at Fe, the product is low-spin Fe^{1+} because of the strong ligand field of the macrocycle.

In this contribution, we use cryoreduction to generate a high-spin, S-ligated Fe^{1+} center within the protein scaffold of apoazurin. The new Fe^{1+} species derives from Fe^{2+} azurin (Fe^{2+}Az), a recently reported metalloprotein in which the natural "blue Cu" center is substituted with Fe^{2+} (Figure 1).[11] The high-spin Fe^{2+} site in Fe^{2+}Az is pseudotetrahedral, with coordination to two His residues, one Cys residue, and the backbone amide O of a Gly. In this study, we show that this Fe^{2+} ion can be reduced to high-spin Fe^{1+} through cryoreduction. To our knowledge, this is the first characterization of high-spin Fe^{1+} in a protein.

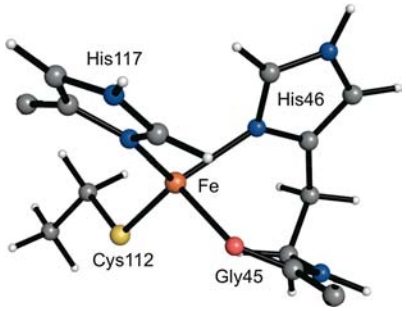

Figure 1. Pseudotetrahedral Fe^{2+} site from substitution of Fe^{2+} for the natural Cu^{2+} ion in *P. aeruginosa* azurin.[11] This is termed Fe^{2+}Az.

A frozen glass of electron paramagnetic resonance (EPR)-silent FeAz (2.5 mM protein, 50 mM MOPS adjusted to pH 7.0, 1 mM DTT, 20% glycerol) was exposed to various doses of γ-rays from ^{60}Co at 77 K. This treatment generated a species with a highly anisotropic EPR signal (Figure 2). The effective g values of this signal indicate that it arises from the lower (l) Kramers doublet of a $S = {}^3/_2$ Fe ion: ${}^{l}\mathbf{g}_{eff} = [5.91, 2.49, 1.66]$ (Figure 2). These effective g values can be described with intrinsic g values for an Fe ion, $\mathbf{g}_{int} \sim [2.0, 2.25, 2.0]$, subject to a zero-field-splitting Hamiltonian with $D > 0$ and $E/D \sim 0.25$.[12] These spin-Hamiltonian parameters can be used to predict the effective g values of the upper (u) doublet of the $S = {}^3/_2$ ion as ${}^{u}\mathbf{g}_{eff} = [5.67, 1.54, 1.36]$. Although the features associated with the lower two g values would be broad and difficult to detect, the third component would be expected to give a sharp signal at a slightly higher field than the signal near 1200 G in Figure 2. Its absence

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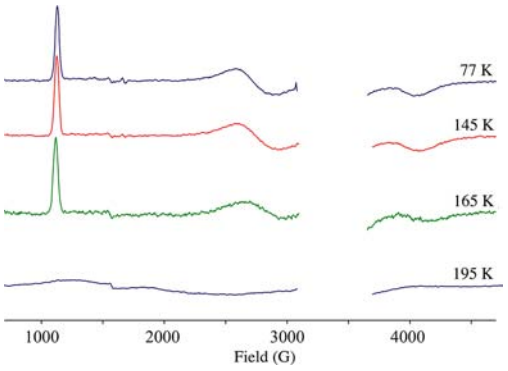

Figure 2. EPR spectra of a frozen 20% glycerol/buffer solution of 2 mM Fe^{2+}Az after exposure to 3.2 Mr of γ irradiation at 77 K and after its annealing at the indicated temperatures. The area near 3400 G is dominated by large signals from radiolytically generated organic radicals and is shown in the SI. Instrument conditions: modulation 100 kHz, modulation amplitude 10 G, microwave power 10 mW, microwave frequency 9.380 GHz, T = 9 K.

indicates that D is sufficiently large that the upper doublet is depopulated at 9 K. Because of rapid spin−lattice relaxation, the intensity of the signal decreases rapidly with increasing temperatures up to 32 K (Figure S2 in the Supporting Information, SI).

The cryogenerated $S = {}^3/_2$ EPR signal was assigned to high-spin Fe^{1+}Az, rather than intermediate-spin Fe^{3+}Az, by two methods. The first is the dose dependence of the intensity of the EPR signal. Figure 3 shows that the yield of this cryogenerated

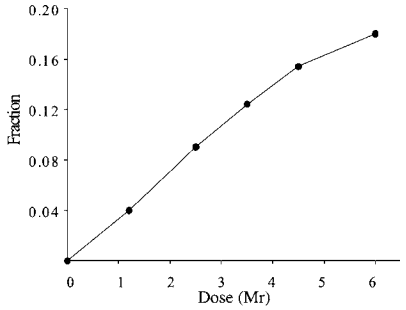

Figure 3. Dose dependence of the intensity of the EPR signal for Fe^{1+}Az.

species increased monotonically with irradiation doses up to 6 Mr. This behavior is expected for cryo*reduction* and contrasts with the typical observation of a maximum in the concentration profile during cryo*oxidation* to Fe^{3+} in hemoproteins.[9] The assignment as a high-spin Fe^{1+} species was also shown by Mössbauer spectroscopy. The Mössbauer spectrum of $^{57}Fe^{2+}$Az in a glycerol/buffered H_2O glass without cryoreduction had δ = 0.90 mm/s and ΔE_Q = 3.17 mm/s, in agreement with the literature spectrum.[11] After cryoreduction, there was 17% conversion to a new species with δ = 1.08 mm/s and ΔE_Q = 1.04 mm/s (Figure 4). The increase in the isomer shift is consistent with a lowering of the the oxidation state of the iron from Fe^{2+} to Fe^{1+} and contrasts with known Fe^{3+} complexes with $S = {}^3/_2$, which have isomer shifts below 0.4 mm/s.[13]

The EPR signal from $S = {}^3/_2$ Fe^{1+}Az is similar to that from $S = {}^3/_2$ Co^{2+} ions of Co^{2+} coordination complexes[14] and Co^{2+} proteins,[15] in which the metal has a distorted tetrahedral environment. We correspondingly assign the high-spin ($S = {}^3/_2$) Fe^{1+} ion as residing in a pseudotetrahedral geometry. Note that a

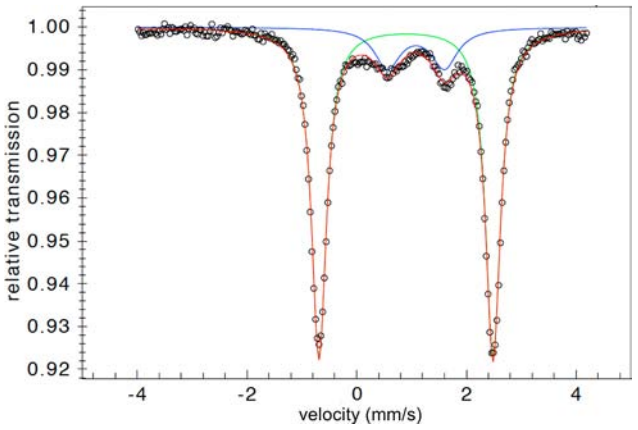

Figure 4. Black circles representing the zero-field Mössbauer spectrum (collected at 80 K) of Fe^{2+}Az after 6 Mr of γ-rays. The red line is from a two-component fit to the spectrum. The relative intensities of the green subspectrum (Fe^{2+}Az) and the blue subspectrum (Fe^{1+}Az) indicate 17% cryoreduction of Fe^{2+}Az to Fe^{1+}Az.

pseudotetrahedral geometry was established for Fe^{2+} in Fe^{2+}Az by use of Mössbauer spectroscopy, crystallography, and quantum-chemical computations.[11] Geometry changes upon cryoreduction normally are minimal,[16] so it is not surprising that the Fe^{1+} analogue is also pseudotetrahedral.

The equilibrium structure of Fe^{1+}Az may, however, differ from that of the species cryogenerated in a frozen solution at 77 K, and such "conformational strain" can be revealed by changes in the EPR signal during structural relaxation upon annealing to higher temperature. Annealing of Fe^{1+}Az at 145 K resulted in only a slight increase in the rhombicity of the EPR signal to $\mathbf{g}_{eff}$ = [5.99, 2.34, 1.62] (Figure 2). Annealing at higher temperature caused a loss of the signal by 175 K without further changes in the effective g values. This may indicate that the equilibrium geometries of Fe^{2+}Az and Fe^{1+} are quite similar or that Fe^{1+}Az decays before it relaxes to its equilibrium geometry. Interestingly, the EPR spectrum of cryogenerated Fe^{1+}Az is different from that for Co^{2+} azurin,[17] despite the similar coordination geometries. Unfortunately, the zero-field-splitting tensor in pseudotetrahedral $S = {}^3/_2$ species is very sensitive to small changes in the coordination environment,[18] so no reliable inferences can be drawn about structural differences from the current data.

The loss of cryogenerated Fe^{1+}Az at relatively low temperature ($T \sim 170$ K) is similar to the behavior of hemoproteins cryoirradiated to give Fe^{1+}.[9] A plausible explanation for the high reactivity of these Fe^{1+} species is that they have very negative $Fe^{2+/1+}$ reduction potentials and are oxidized easily by radiolytically generated matrix radicals or even matrix molecules themselves. This is consistent with the observation that Fe^{2+}Az could not be reduced using protein film voltammetry or with very strong reductants like Eu^{2+}-DTPA.[11]

Though many "blue Cu" sites are characterized by interaction of the Cu ion with a nearby methionine residue, the corresponding Met121 is relatively distant from the metal in Cu^{2+}-azurin,[19] and Mössbauer and computational studies showed that it does not coordinate at all in Fe^{2+}Az.[11] Thus, it is interesting that cryoreduction of the Fe^{2+} complex of azurin with a Met121Ala mutation[11] yielded *no* metal-based EPR signals after an equivalent dose of γ-rays. We speculate that small geometric changes around the Fe site in the mutant could further lower the $Fe^{2+/1+}$ reduction potential, rendering cryoreduction

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ineffective. Future studies on a broader set of mutant proteins are necessary to explore these interesting differences more fully.

In conclusion, cryoreduction of the non-heme Fe^{2+} adduct of the azurin protein generates a species with EPR and Mössbauer characteristics of low-potential, high-spin Fe^{1+}. We anticipate that this approach will be useful in characterizing natural or engineered Fe^{1+} sites in highly reduced biological systems.

■ ASSOCIATED CONTENT

Supporting Information

Additional EPR and Mössbauer spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) (a) Beinert, H.; Holm, R. H.; Münck, E. *Science* **1997**, *277*, 653. (b) Fontecave, M. *Nat. Chem. Biol.* **2006**, *2*, 171.

(2) (a) Burgess, B. K.; Lowe, D. J. *Chem. Rev.* **1996**, *96*, 2983. (b) Howard, J. B.; Rees, D. C. *Chem. Rev.* **1996**, *96*, 2965. (c) Smith, B. E. *Adv. Inorg. Chem.* **1999**, *47*, 159. (d) Tezcan, F. A.; Kaiser, J. T.; Mustafi, D.; Walton, M. Y.; Howard, J. B.; Rees, D. C. *Science* **2005**, *309*, 1377. (e) Spatzal, T.; Aksoyoglu, M.; Zhang, L.; Andrade, S. L. A.; Schleicher, E.; Weber, S.; Rees, D. C.; Einsle, O. *Science* **2011**, *334*, 940. (f) Wiig, J. A.; Hu, Y.; Lee, C. C.; Ribbe, M. W. *Science* **2012**, *337*, 1672.

(3) (a) Guo, M.; Sulc, F.; Ribbe, M. W.; Farmer, P. J.; Burgess, B. K. *J. Am. Chem. Soc.* **2002**, *124*, 12100. (b) Deng, L.; Holm, R. H. *J. Am. Chem. Soc.* **2008**, *130*, 9878. (c) Münck, E.; Bominaar, E. L. *Science* **2008**, *321*, 1452. (d) Bill, E. *Hyperfine Interact.* **2012**, *205*, 139. (e) Albers, A.; Demeshko, S.; Pröpper, K.; Dechert, S.; Bill, E.; Meyer, F. *J. Am. Chem. Soc.* **2013**, *135*, 1704.

(4) (a) Siegbahn, P. E. M.; Westerberg, J.; Svensson, M.; Crabtree, R. H. *J. Phys. Chem. B* **1998**, *102*, 1615−1623. (b) Hoffman, B. M.; Lukoyanov, D.; Dean, D. R.; Seefeldt, L. C. *Acc. Chem. Res.* **2013**, *46*, 587.

(5) (a) Brown, S. D.; Betley, T. A.; Peters, J. C. *J. Am. Chem. Soc.* **2003**, *125*, 322. (b) Hendrich, M. P.; Gunderson, W.; Behan, R. K.; Green, M. T.; Mehn, M. P.; Betley, T. A.; Lu, C. C.; Peters, J. C. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 17107. (c) Smith, J. M.; Sadique, A. R.; Cundari, T. R.; Rodgers, K. R.; Lukat-Rodgers, G.; Lachicotte, R. J.; Flaschenriem, C. J.; Vela, J.; Holland, P. L. *J. Am. Chem. Soc.* **2006**, *128*, 756. (d) Yu, Y.; Smith, J. M.; Flaschenriem, C. J.; Holland, P. L. *Inorg. Chem.* **2006**, *45*, 5742. (e) Chiang, K. P.; Scarborough, C. C.; Horitani, M.; Lees, N. S.; Ding, K.; Dugan, T. R.; Brennessel, W. W.; Bill, E.; Hoffman, B. M.; Holland, P. L. *Angew. Chem., Int. Ed.* **2012**, *51*, 3658.

(6) (a) Mock, M. T.; Popescu, C. V.; Yap, G. P. A.; Dougherty, W. G.; Riordan, C. G. *Inorg. Chem.* **2008**, *47*, 1889. (b) Rodriguez, M. M.; Stubbert, B. D.; Scarborough, C. C.; Brennessel, W. W.; Bill, E.; Holland, P. L. *Angew. Chem., Int. Ed.* **2012**, *51*, 8247. (c) In a recent report, reduction of an $Fe^{2+}-N_2$ complex with S-based ligands gave a dinuclear complex, with one of the sites most likely being low-spin Fe^{1+}: Takaoka, A.; Mankad, N. P.; Peters, J. C. *J. Am. Chem. Soc.* **2011**, *133*, 8440. (d) Reduction of the three-coordinate iron(2+) thiolate complex $L^{tBu}FeSPh$ gives S−C bond cleavage: Chiang, K. P.; Kaplan, E.; Holland, P. L., unpublished results.

(7) For a few recent examples, see: (a) Jablonskytė, A.; Wright, J. A.; Fairhurst, S. A.; Peck, J. N. T.; Ibrahim, S. K.; Oganesyan, V. S.; Pickett, C. J. *J. Am. Chem. Soc.* **2011**, *133*, 18606. (b) Erdem, Ö. F.; Schwartz, L.; Stein, M.; Silakov, A.; Kaur-Ghumaan, S.; Huang, P.; Ott, S.; Reijerse, E. J.; Lubitz, W. *Angew. Chem., Int. Ed.* **2011**, *50*, 1439. (c) Adamska, A.; Silakov, A.; Lambertz, C.; Rüdiger, O.; Happe, T.; Reijerse, E.; Lubitz, W. *Angew. Chem., Int. Ed.* **2012**, *51*, 11458.

(8) (a) Denisov, I. G.; Makris, T. M.; Sugar, S. G. *Methods Enzymol.* **2002**, *357*, 103. (b) Telser, J.; Davydov, R.; Horng, Y.-C.; Ragsdale, S. W.; Hoffman, B. M. *J. Am. Chem. Soc.* **2001**, *123*, 5853−5860. (c) Davydov, R.; Behrouzian, B.; Smoukov, S.; Stubbe, J.; Hoffman, B. M.; Shanklin, J. *Biochemistry* **2005**, *44*, 1309−1315.

(9) Davydov, R.; Hoffman, B. *J. Biol. Inorg. Chem.* **2008**, *13*, 357.

(10) (a) Mot, A.; Zoltan, K.; Svistunenko, D. A.; Damian, G.; Silaghi-Dumitrescu, R.; Makarov, S. V. *Dalton Trans.* **2010**, *39*, 1464. (b) Grodkowski, J.; Behar, D.; Neta, P.; Hambright, P. *J. Phys. Chem. A* **1997**, *101*, 248. (c) Grodkowski, J.; Dhanasekaran, T.; Neta, P.; Hambright, P.; Brunschwig, B. S.; Shinozaki, K.; Fujita, E. *J. Phys. Chem. A* **2000**, *104*, 11332. (d) Nevin, W. A.; Liu, W.; Melník, M.; Lever, A. B. P. *J. Electroanal. Chem.* **1986**, *213*, 217.

(11) (a) McLaughlin, M. P.; Retegan, M.; Bill, E.; Payne, T. M.; Shafaat, H. S.; Peña, S.; Sudhamsu, J.; Ensign, A. A.; Crane, B. R.; Neese, F.; Holland, P. L. *J. Am. Chem. Soc.* **2012**, *134*, 19746. (b) Kolczak, U.; Dennison, C.; Messerschmidt, A.; Canters, G. W. Azurin and Azurin Mutants. In *Handbook of Metalloproteins*; Wiley: New York, 2001; Vol. *2*, p 1170. (c) Lu, Y. Cupredoxins. In *Comprehensive Coordination Chemistry 2*; McCleverty, J., Meyer, T. J. , Eds.; Elsevier: Oxford, 2004; Vol. *8*.

(12) Hoffman, B. M.; Weschler, C. J.; Basolo, F. *J. Am. Chem. Soc.* **1976**, *98*, 5473.

(13) (a) Münck, E. Aspects of ^{57}Fe Mössbauer Spectroscopy. In *Physical Methods in Bioinorganic Chemistry*; Que, L., Ed.; University Science Books: Sausalito, CA, 2000; p 287. (b) Gütlich, P.; Bill, E.; Trautwein, A. X. *Mössbauer Spectroscopy and Transition Metal Chemistry*; Springer Verlag: Berlin, 2011.

(14) For examples, see: (a) Yablokov, Y. V.; Voronkova, V. K.; Shishkov, V. F.; Ablov, A. V.; Vaisbein, Z. Y. *Fiz. Tverd. Tela* **1971**, *13*, 997. (b) Maganas, D.; Milikisyants, S.; Rijnbeek, J. M. A.; Sottini, S.; Levesanos, N.; Kyritsis, P.; Groenen, E. J. J. *Inorg. Chem.* **2009**, *49*, 595.

(15) (a) Cockle, S. A.; Lindskog, S.; Grell, E. *Biochem. J.* **1974**, *143*, 703. (b) Mathies, G.; Almeida, R. M.; Gast, P.; Moura, J. J. G.; Groenen, E. J. J. *J. Phys. Chem. B* **2012**, *116*, 7122.

(16) (a) Davydov, R.; Kuprin, S.; Gräslund, A.; Ehrenberg, A. *J. Am. Chem. Soc.* **1994**, *116*, 11120. (b) Davydov, R.; Valentine, A. M.; Komar-Panicucci, S.; Hoffman, B. M.; Lippard, S. J. *Biochemistry* **1999**, *38*, 4188. (c) Davydov, R.; Makris, T. M.; Kofman, V.; Werst, D. E.; Sligar, S. G.; Hoffman, B. M. *J. Am. Chem. Soc.* **2001**, *123*, 1403.

(17) Bonander, N.; Vanngard, T.; Tsai, L.-C.; Langer, V.; Nar, H.; Sjoelin, L. *Proteins* **1997**, *27*, 385.

(18) (a) Krzystek, J.; Swenson, D. C.; Zvyagin, S. A.; Smirnov, D.; Ozarowski, A.; Telser, J. *J. Am. Chem. Soc.* **2010**, *132*, 5241. (b) Maganas, D.; Sottini, S.; Kyritsis, P.; Groenen, E. J. J.; Neese, F. *Inorg. Chem.* **2011**, *50*, 8741.

(19) Crane, B. R.; Di Bilio, A. J.; Winkler, J. R.; Gray, H. B. *J. Am. Chem. Soc.* **2001**, *123*, 11623.

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