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

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Abstract

High-spin Fe¹⁺ 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 Fe¹⁺ in a protein environment. Cryoreduction of Fe²⁺-substituted azurin at 77 K with ⁶⁰Co radiation generates a new species with a *S* = 3/2 (high-spin) Fe¹⁺ center having *D* > 0 and *E/D* ~ 0.25. This transient species is stable in a glycerol-water glass only up to ~170 K. A combination of EPR and Mössbauer spectroscopies provides a powerful means of identifying a transient high-spin Fe¹⁺ site in a protein scaffold.

Keywords

Azurin; Iron; Radiolytic reduction; Redox; EPR; Mössbauer

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 potential¹ and for reduction of challenging substrates like N₂.² Highly reduced FeS clusters include all-Fe²⁺ FeS clusters and synthetic analogues.³ The next reduction step beyond all-Fe²⁺ would generate subferrous centers with high-spin Fe¹⁺. The generation of super-reduced FeS intermediates may underlie the binding and activation of N₂ by FeMoco, the active site of nitrogenase.⁴ Few high-spin Fe¹⁺ complexes are known,⁵ with the first example of a synthetic high-spin Fe¹⁺ complex supported by any S donor having been described recently.⁶

Low-spin Fe¹⁺ sites, on the other hand, have been characterized in detail, particularly in the iron-only hydrogenase enzymes.⁷ Because the understanding of high-spin Fe¹⁺ has lagged behind that of low-spin Fe¹⁺, it remains a challenge to discover the key identifying characteristics of high-spin Fe¹⁺, 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

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ASSOCIATED CONTENT

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

has been used most commonly to activate oxy-ferrous monooxygenases, but has also been shown to reduce some hemoproteins from Fe^{2+} to formally Fe^{1+} states.⁹ Radiolytic reduction and chemical reduction have also been used to reduce synthetic phthalocyanine- Fe^{2+} complexes.¹⁰ In the previous cases where reduction occurs at iron, the site has low-spin Fe^{1+} because of the strong ligand field of the macrocycle.

In this contribution, we use cryoreduction to generate a high-spin, sulfur-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 copper” 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 oxygen 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.

A frozen glass of 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 (Fig 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: $^l g_{\text{eff}} = [5.91, 2.49, 1.66]$ (Fig 2). These effective g -values can be described with intrinsic g -values for an Fe ion, $g_{\text{int}} \sim [2.0, 2.25, 2.0]$, 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 $^u g_{\text{eff}} = [5.67, 1.54, 1.36]$. Although the features associated with the lower two g -values would be broad and difficult to detect, one component would be expected to give a sharp signal at a slightly higher field than the signal near 1200 G in Fig 2. Its absence 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 (Fig. S2).

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. Fig 3 shows that the yield of this cryogenerated species increased monotonically with irradiation doses up to 6 Mr. This behavior is expected for cryoreduction, and contrasts with the typical observation of a maximum in the concentration profile during cryooxidation 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 $^{57}\text{Fe}^{2+}\text{Az}$ in a glycerol/buffered H_2O glass without cryoreduction had $\delta = 0.90$ mm/s and $E_Q = 3.17$ mm/s, in agreement with the literature spectrum in buffer.¹¹ After cryoreduction, there was 17% conversion to a new species with $\delta = 1.08$ mm/s and $E_Q = 1.04$ mm/s (Fig 4). The increase in the isomer shift is consistent with lowering 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 of the $S = 3/2$ Co^{2+} ions of Co^{2+} coordination complexes¹⁴ and Co^{2+} proteins¹⁵ 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 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 the Fe^{1+}Az may, however, differ from that of the species cryogenerated in 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 $g_{\text{eff}} = [5.99, 2.34, 1.62]$ (Fig 2). Annealing at higher temperature caused loss of the signal by 175 K without further change 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 $\text{Fe}^{2+/1+}$ 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 copper” sites are characterized by an interaction of the copper 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 the cryoreduction of the Fe^{2+} complex of azurin with a Met121Ala mutation¹¹ yielded *no* metal-based EPR signals after an equivalent dose of γ -rays. We speculate that small geometric changes around the iron site in the mutant could further lower the $\text{Fe}^{2+/1+}$ reduction potential, rendering cryoreduction ineffective. Future studies on a broader set of mutant proteins is necessary to explore these interesting differences more fully.

In conclusion, cryoreduction of the Fe^{2+} adduct of the azurin protein generates a species with EPR and Mössbauer characteristics that identify it as 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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SYNOPSIS

The Fe^{2+} complex of the azurin apoprotein can be reduced by gamma rays at low temperature to give a pseudotetrahedral high-spin Fe^{1+} site in the metalloprotein, as shown by a combination of EPR and Mössbauer spectroscopies.

Figure 1.
The 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 .

Figure 2.

EPR spectra of 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 Supporting Information. Instrument conditions: 100 kHz modulation, modulation amplitude 10 G, microwave power 10 mW, microwave frequency 9.380 GHz, $T = 9$ K.

Figure 3.
Dose dependence of the intensity of the EPR signal for Fe¹⁺Az.

Figure 4.

The black circles represent 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 the Fe^{2+}Az to Fe^{1+}Az .