THE ELECTRON PARAMAGNETIC RESONANCE OF OXIDIZED CLOSTRIDIAL FERREDOXINS

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SUMMARY: A weak EPR resonance at g = 2.015 has been observed in five oxidized Clostridial-type ferredoxins. Oxidized *Clostridium acidi-urici* [^{57}Fe] ferredoxin exhibited a broadened resonance at g = 2.015 relative to the signal from native or reconstituted [^{56}Fe]ferredoxin. This experiment, in conjunction with other results, demonstrates that the signal exhibited by oxidized Clostridial ferredoxin either originates from the $Fe_4S_4^*$ clusters of the protein or from a paramagnetic species bound to the clusters. Oxidized *C. acidi-urici* ferredoxin, when treated with the oxidant potassium ferricyanide, was found to exhibit an approximate 100 fold increase in the intensity of the signal. Results of experiments performed on the ferricyanide treated ferredoxin are consistent with the existence of a third oxidation state of the protein.

Iron-sulfur proteins function as essential electron carriers in a wide variety of bacterial, plant, and animal cells (1). The electron paramagnetic resonance (EPR) signal of reduced iron-sulfur proteins at g = 1.94 has been of major importance in studies (2) in this relatively recently recognized class of proteins (3). Clostridial-type ferredoxins, which contain two cube-like $Fe_4S_4^*$ clusters, exhibit in their reduced form a complex EPR signal centered about g = 1.94 that has been shown to result from two weakly coupled electronic spins of S = 1/2 (4). The EPR signal of the oxidized form of this protein is much weaker, and differs from that of the reduced protein (5,6). This signal, in contrast to that of the reduced protein, has received little attention and its significance and possible relation to the biological function of the protein has not been established.

The magnitude of the signal of oxidized Clostridial ferredoxin that we have observed varies with the protein preparation and is small (<0.02 spins/molecule as determined by quantitative EPR experiments). The resonance occurs at g = 2.015 with a linewidth of approximately 30 gauss and is visible only at temperatures below about 35 K. It appears to consist of two overlapping resonances and is saturated at even lower microwave powers than is the reduced signal.

This resonance at g = 2.015 has been observed in the oxidized form of a number of iron-sulfur proteins. These proteins have been isolated from a variety of

sources using a number of different isolation procedures. Five $(Fe_4S_4^*)_2$ ferredoxins (Clostridial-type) examined in this laboratory exhibit the signal. In addition, a similar resonance has been reported in the following iron-sulfur proteins, all of which contain at least four iron and four inorganic sulfur atoms: *Bacillus polymyxa* $Fe_4S_4^*$ ferredoxin I (7); an iron-sulfur protein from *Azotobacter vinelandii* (8); nitrate reductase A from *Micrococcus denitrificans* (9); and NADH dehydrogenase from *Azotobacter vinelandii* (10). The resonance has also been observed in two proteins whose iron-sulfur content has not yet been established: adenylyl sulfate reductase from *Desulfovibrio vulgaris* (11) and ubihydroquinone-cytochrome c reductase (complex III) (12, 13). This resonance has not, however, been observed in any well characterized protein containing less than four iron and four inorganic sulfur atoms.

The presence of this EPR resonance in so many different iron-sulfur proteins suggests that it is a significant property of these proteins and may be involved in their biological function. A series of experiments have been performed using *C. acidi-urici* ferredoxin in an attempt to determine the origin of this unidentified resonance.

Iron and inorganic sulfide were removed from *C. acidi-urici* ferredoxin and the protein was reconstituted with ^{57}Fe and sulfide according to the methods previously described (14,15). A second sample of reconstituted ferredoxin was prepared with ^{56}Fe and sulfide. The spectrum of the oxidized form of the [^{57}Fe] ferredoxin was broadened relative to that of the native or reconstituted [^{56}Fe] ferredoxin by approximately 10 gauss. This broadening is due to unresolved hyperfine splitting from the ^{57}Fe nucleus (16), and indicates that the unpaired electron generating the EPR resonance is physically close to one or more iron atoms. Three possible origins for the signal have been considered. The signal could come from an iron-sulfur cluster in the protein, it could result from a free radical coupled to the iron-sulfur cluster, or it could arise from protein bound iron originating from denatured ferredoxin.

Analysis for the types of iron species could give rise to an EPR resonance similar to that exhibited by oxidized Clostridial ferredoxin was performed. This

is useful in evaluating the possibility that the signal arises from protein-bound iron derived from denatured ferredoxin. There are only two states of iron in which a single atom could exhibit a signal of this type: low spin Fe(III) and high spin Fe(III) (17). Both these ions, however, would require a ligand field of near perfect cubic symmetry. Such a ligand field on the surface of a protein is not probable. Considering the wide variety of iron-sulfur proteins that exhibit the signal, a single protein bound iron atom is considered an unlikely source of the resonance. The possibility of two or more adventive irons binding in a specific manner to so many different proteins also seems unlikely. To test the possibility of the signal arising from adventive iron, two experiments were performed. In the first experiment, *C. acidi-urici* ferredoxin was denatured with heat at 65 C and ESR spectra of samples treated for various times up to 150 min were examined. The decrease in the A_{390}/A_{280} ratio of the ferredoxin was accompanied by a decrease in the intensity of the g = 2.015 signal. This is inconsistent with the signal originating from iron bound to the apoprotein, but it does not rule out the possibility of a binding site for iron on the surface of the intact ferredoxin molecules. In the second experiment, the possibility of iron originating from denatured protein and then binding to native protein was tested. Aliquots of increasing amounts of the mixture of Fe(II) and Na_2S used for reconstitution were added to a solution of native ferredoxin (1.85×10^{-4} M, 30 mM Tris-chloride buffer, pH 7.4) to see if the EPR signal increased in intensity. No increase in the EPR signal intensity was observed for concentrations of Fe(II) and Na_2S of up to two fold greater than the concentration of ferredoxin. These results, in conjunction with the symmetry requirement for any single iron source of the signal, imply that the resonance observed in oxidized Clostridial ferredoxin must either originate from the $Fe_4S_4^*$ clusters themselves or from a free radical bound to the clusters.

If the signal does in fact originate from the iron-sulfur clusters of the protein, then it is possible that it arises from a small population of clusters in an oxidation state above what is normally considered oxidized ferredoxin.

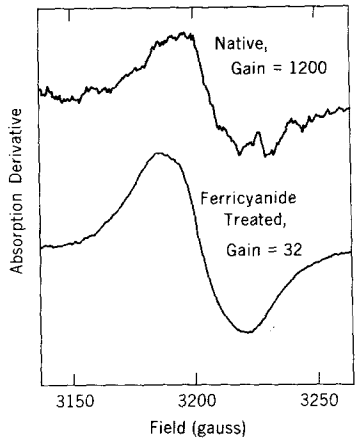

Fig. 1. Frozen solution X-band EPR spectra of native and potassium ferricyanide treated *Clostridium acidi-urici* ferredoxin. Power to the cavity, 2 mw; field modulation, 2 gauss; temperature, 15 K. Ferricyanide treated ferredoxin: A_{390}/A_{280} = 0.632, 1.44 x 10^{-5} M, in 0.1 M Tris-chloride buffer, pH 7.4, gain = 32. Native ferredoxin: A_{390}/A_{280} = 0.796, 2.84 x 10^{-3} M, in 0.1 M Tris-chloride buffer, pH 7.4, gain = 1200. The EPR spectra were recorded with a modified JEOL ME-1X Spectrometer with a TE_{011}-mode cylindrical cavity. A magnetic field modulation of 100 KHz was employed.

According to the three-state theory of Carter, *et al.* (18), such a super-oxidized cluster would be formally equivalent to the iron-sulfur cluster in oxidized high-potential iron protein (HiPIP). This hypothesis was tested by attempting to super-oxidize ferredoxin using a number of different chemical oxidants. One of these, $K_3Fe(CN)_6$, produced an approximate 100 fold enhancement of the g = 2.015 resonance. Reaction with this oxidant is slow, and destructive side reactions are competitive. A short purification procedure with Sephadex G-25, DEAE-cellulose, and ammonium sulfate precipitation is used to separate the ferredoxin from the ferricyanide. The EPR signal of the super-oxidized ferredoxin obtained in this way has the same g-value, a similar line width, and the same general shape as the signal from the untreated ferredoxin (Fig. 1). The signal from ferricyanide treated ferredoxin, however, was less easily saturated than the signal from native ferredoxin.

Quantitative EPR experiments on the ferricyanide treated ferredoxin show the presence of 1.5 unpaired spins/molecule, based on a molar extinction coefficient

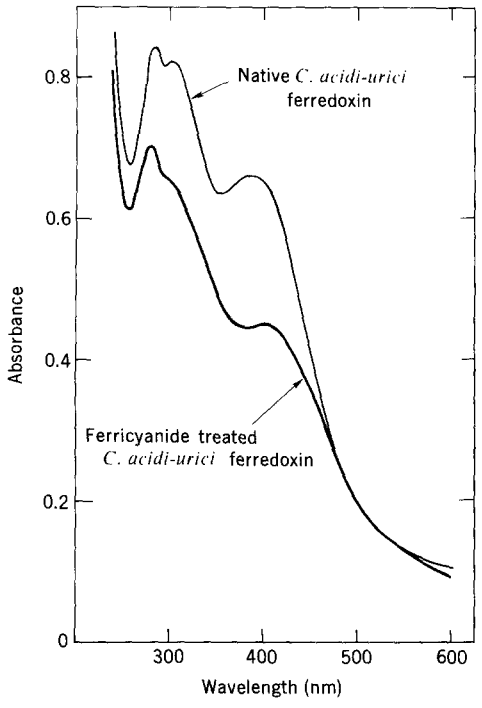

Fig. 2. Optical absorption spectra of native and potassium ferricyanide treated *Clostridium acidi-urici* ferredoxins.

of ε_{390} = 30,600. If ferricyanide super-oxidizes both iron-sulfur clusters in the protein, the fully super-oxidized material would be expected to show 2.0 spins per molecule. The discrepancy between the 1.5 spins observed and the 2.0 spins expected might result from the incomplete reaction with ferricyanide, the presence of impurities contributing to the absorption at 390 nm, or to a different value of ε_{390} for the fully reacted material.

The optical spectrum of the ferricyanide treated ferredoxin is similar to that of native ferredoxin (Fig. 2), but has an A_{390}/A_{280} ratio of approximately 0.6 compared to a value of 0.79 for the native protein. The peak at 390 nm is red shifted to about 410 nm. At this time it is not clear whether these spectral differences are caused by the presence of other reaction products after reaction with ferricyanide or to a real difference in the absorption spectrum of ferricyanide treated ferredoxin from that of native ferredoxin.

Ferricyanide treated ferredoxin has been found by optical examination to be reduced to the same extent as native ferredoxin by dithionite, hydrogen-hydrogenase (19), and by the phosphoroclastic system (20). In addition, no difference in activity between native and ferricyanide treated ferredoxin was found in the phosphoroclastic assay (21,22) assuming both proteins to have the same molar extinction coefficient at 390 nm.

It has been shown that the EPR signal observe in oxidized Clostridial ferredoxins originates either from a $Fe_4S_4^*$ cluster or from a paramagnetic species bound to a $Fe_4S_4^*$ cluster. The experiments described make the possibility of a super-oxidized $Fe_4S_4^*$ cluster an attractive explanation for the EPR signal.

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