HETEROGENEITY OF PARAMAGNETIC SPECIES IN TWO IRON-SULFUR PROTEINS: CLOSTRIDIUM PASTEURIANUM FERREDOXIN AND MILK XANTHINE OXIDASE*

W. H. Orme-Johnson and Helmut Beinert

Institute for Enzyme Research, University of Wisconsin
1710 University Avenue, Madison, Wisconsin, 53706

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During the anaerobic reductive titration of both C. pasteurianum ferredoxin and milk xanthine oxidase, two different EPR signals ascribable to iron were seen to arise in succession. More than one species of iron is therefore involved in electron transfer in each one of these proteins.

The iron-sulfur proteins which contain two iron atoms and two acid-labile sulfur atoms were without exception found to accept one electron on reduction at neutral pH and room temperature, and this electron is quantitatively accounted for in the EPR spectrum (1 - 5). For two members of this class, putidaredoxin and the adrenal iron-sulfur protein, nuclear hyperfine interaction studies showed that the unpaired electron interacts with both iron and both labile sulfur atoms, which must therefore exist in a single functional paramagnetic center in these proteins (1, 2, 5, 6).

In a number of cases, e.g. the bacterial ferredoxins, aldehyde and xanthine oxidases, succinate and dihydroorotate dehydrogenases, and the high potential iron-sulfur protein from Chromatium, up to eight iron and eight labile sulfur atoms per molecule (7 - 11) may be present. With the exception of the Chromatium protein, these more complex iron-sulfur proteins may take up more than one electron on reduction (12 - 16). It is of interest, therefore, to know whether the iron sites present are of one kind only or are heterogeneous. Evidence for heterogeneity of the iron components of more complex iron-sulfur

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proteins has in fact been previously available. First, the observation that clostridial ferredoxin is only able to take up one or two electrons on reduction, although 7 - 8 iron atoms are present suggests (but does not prove) heterogeneity. Secondly, kinetic as well as reductive titration studies on aldehyde oxidase (13) and on succinate dehydrogenase (16) clearly showed that the iron components of these proteins do not react as would be expected of a single species. Thirdly, the Mössbauer (17) as well as EPR spectra (18) of clostridial ferredoxin showed a complexity which suggested heterogeneity in either the population of molecules or in the iron components within individual molecules. Except for these last observations, previous evidence of heterogeneity was based on the fact that only a fraction of the total iron sites could be directly observed to undergo a certain reaction, while the remainder was not unambiguously detectable. Although this would appear to be a valid criterion of heterogeneity, we have now observed directly by EPR spectroscopy the appearance, in succession, of two paramagnetic species in clostridial ferredoxin and xanthine oxidase. Although xanthine oxidase contains flavin and molybdemum in addition to iron, there is very little doubt that the observed paramagnetic species represent iron sites of these proteins.

Ferredoxin

Determinations of the number of electrons taken up by C. pasteurianum ferredoxin on reduction have been reported by Sobel and Lovenberg (12) and by Tagawa and Arnon (19) using the hydrogen-hydrogenase system as reductant. The former authors found that two electrons are transferred, while the latter authors found a one-electron transfer. Palmer and coworkers (18) described an EPR signal in reduced ferredoxin which is well developed only below $\sim 30^{\circ}$ K. With these findings in mind, we sought to apply to this problem a technique of anaerobic reduction with solid-diluted dithionite, that has been useful with the simpler iron-sulfur proteins, succinate dehydrogenase, and cytochrome oxidase (1, 2, 5, 16, 20).

The ferredoxin used here was prepared by either a procedure due to Hardy

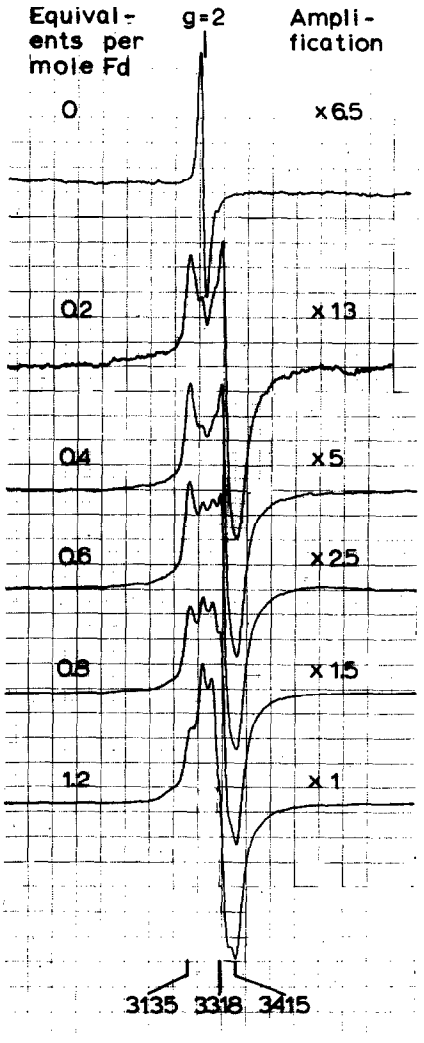

FIGURE 1: EPR spectra (first derivative of absorption) observed during anaerobic titration of clostridial ferredoxin with dithionite. The EPR tubes initially contained 0.300 ml of a solution, 0.43 mM in ferredoxin and 0.1 M in potassium phosphate, at pH 7.0. The sample volume decreased 10 - 17% during evacuation and flushing with helium; the amplitudes of the curves shown here are not adjusted to reflect the small differences among tubes due to this effect. Graded amounts of solid diluted (24 neq/mg) sodium dithionite were added as elsewhere described (25), after anaerobiosis had been achieved. The number of reducing equivalents added per mole of protein are indicated on the left hand side, the amplification used in recording the spectra is shown on the right hand side relative to the spectrum on the bottom line. No significant change in signal shape was observed after the addition of 1.2 equivalents of reductant. The conditions of EPR spectroscopy were: microwave power, 3 mwatt; modulation amplitude, 12 gauss; scanning rate, 1000 gauss per min, time constant, 0.5 sec; and temperature, 20° K. The frequency was 9,071 MHz. The principal peaks are indicated by markers in gauss.

et al (21) whereby the protein is extracted with buffer from dried cells under hydrogen, followed by heat precipitation, DEAE and Sephadex G-100 chromatography and crystallization, or by the acetone extraction procedure of Mortenson (22). In both cases the results to be described were obtained; the majority of the studies were with the former preparation. A sample of this protein was desalted for analytical purposes on Sephadex G-25 and was found to contain, per 6.0 mg protein (dry weight), 7.3 μmoles of acid-labile sulfur [determined as in (23)] and 8.4 μg atoms of total iron [determined as in (24)], and assuming a molecular weight of 6,000 (22), an $E_{mM} = 24$ at 390 mμ. The ratio A_{390}/A_{280} was 0.79.

When aliquots of ferredoxin were placed in EPR tubes, made anaerobic and reduced with graded amounts of solid-diluted dithionite[1] (25), frozen after ca. 5 minutes and examined at 20° K, the changes in the EPR spectrum depicted in Figure 1 were seen. The small signal near g = 2 in the starting protein was replaced during early additions of reducing equivalents by a signal with $g_{\parallel} \sim 2.06$, $g_{\perp} \sim 1.94$. As further equivalents were added, this signal increased in size and at the same time there arose a narrower signal in the center of the original spectrum. All spectral changes of both size and shape were complete when two reducing equivalents per molecule had been added. A similar sequence of changes was seen when ferredoxin was incubated anaerobically with hydrogenase and increasing pressures of hydrogen, although complete reduction was not achieved under the conditions used (pH 7, <900 mm Hg H_2 pressure) (cf. 19).

Xanthine Oxidase

Xanthine oxidase from milk contains two molybdenum and eight iron atoms and two flavin residues per protein molecule (26). Reductive titrations with substrates are complicated by the size of the substrate binding constants and by slow (possibly secondary) changes of the molybdenum signals (15, 27). We sought to circumvent these problems with a dithionite titration in the presence of methyl viologen as mediator, a procedure that was sucessful with the

[1] This procedure has been partly described previously (1, 13), and will be published in detail elsewhere (25).

reductively sluggish adrenal iron-sulfur protein (5). Preliminary experiments indicated that in the presence of 10 mol % (protein molarity) benzyl viologen, rapid changes in EPR spectra of xanthine oxidase, partly reduced by dithionite, were complete within one minute, while no "slow" molybdenum signal (28) was seen.

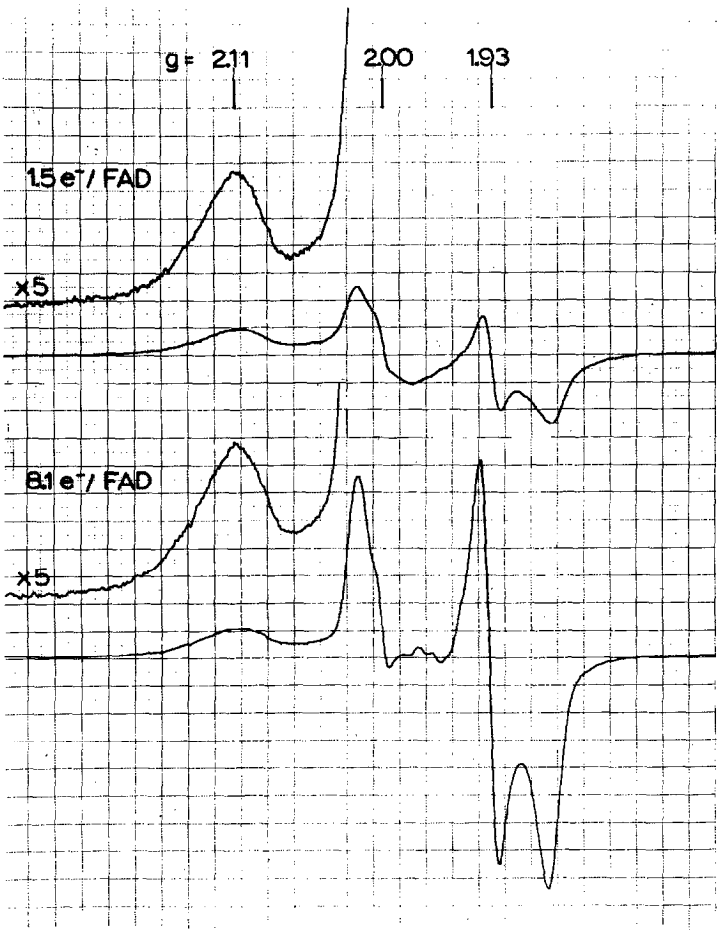

FIGURE 2: EPR spectra observed during anaerobic titration of milk xanthine oxidase with solid dithionite. 1.5 reducing equivalents per mole of enzyme bound FAD (½ mole of protein) was added to give the top pair of spectra, while the addition of 8.1 equivalents per mole FAD led to the bottom pair of spectra. A segment of the spectrum with the gain augmented 5-fold is shown so that the g = 2.11 resonance may be more clearly seen. The conditions of EPR spectroscopy were: microwave power, 0.3 mwatt; modulation amplitude, 5 gauss; scanning rate, 400 gauss per min; time constant, 0.5 sec; temperature, 13° K; and frequency, 9,233 MHz. The EPR cuvettes initially held 0.400 ml of a solution containing 0.061 mM xanthine oxidase (0.122 mM FAD), 0.1 M Tris hydrochloride, and 0.01 mM benzylviologen, at pH 7.9. The procedure used to produce reduced states of the enzyme were as described in the legend to Figure 1.

The milk xanthine oxidase used in these studies was the gift of Dr. R. C. Bray, and had, per flavin molecule, 1.0 atom of molybdenum and 4.0 atoms of iron.

When an anaerobic reductive titration including the mediator but otherwise analogous to that of ferredoxin was carried out, the iron signal with the g values $g_x = 1.899$; $g_y = 1.935$ and $g_z = 2.022$ (29), which can be observed at liquid nitrogen temperature (27, 29), was maximally present only after 8 reducing equivalents had been added. In contrast, the EPR signal at g = 2.11 reached maximal intensity when 3 reducing equivalents per molecule had been added as shown in Figure 2. This signal, which had previously been observed by Gibson and Bray (29) and by Palmer and Massey (27), is only detectable at low temperatures (<30° K) and is also presumed to be due to iron.

Discussion

Although the EPR signals observed here with the more complex iron sulfur proteins have not yet been definitively identified as signals stemming from iron complexes, it seems reasonable to assume from the studies on the simpler iron-sulfur proteins (1 - 6) that this is indeed the case. Since these signals arise separately and additively during reductive titration they must represent distinct paramagnetic iron-complexes in the molecule[2], unless a heterogeneous population of molecules is postulated. These separate sites may of course have functionally different roles. It may also be inferred that if the different paramagnetic centers in bacterial ferredoxin and milk xanthine oxidase were in thermodynamic equilibrium in these experiments, then they have different oxidation-reduction potentials, which in turn may reflect as yet unknown differences in function.

It would be important to be able to give a quantitative estimate of the numbers of unpaired electrons represented by each of the signals described, as has been done in the case of the simpler iron-sulfur proteins (cf. 1, 2, 5).

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[2] It has been found by D. Ballou, G. Palmer and V. Massey (personal communication) that the two signals in xanthine oxidase arise in a kinetically distinct manner on reduction by substrates.

Because of the strong temperature dependence (18, 27, 29) and spectral complexity of these signals it will require considerably more work to arrive at reliable estimates. In the case of ferredoxin we can place an upper limit on the number of electrons represented, namely two, and since both signals represent a roughly equal number of electrons, it would seem reasonable to assign one electron to each center. It must be remembered in this context that, according to the model of the simpler iron-sulfur proteins, one electron is taken up by a two-iron center. If this model holds for the more complex ferredoxins, then, our observation by EPR spectroscopy of two different iron species, which together only account for uptake of two electrons, whereas the protein contains eight per molecule, indicates that there is at least one more species of iron present, which remains undetectable by EPR. Further studies on this subject will be presented and discussed more fully elsewhere.

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