*THE NUMBER OF IRON ATOMS IN THE PARAMAGNETIC CENTER (G = 1.94) OF REDUCED PUTIDAREDOXIN, A NONHEME IRON PROTEIN**

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A number of oxidation-reduction proteins, containing iron and an acid-labile form of sulfide, have been shown to exhibit low-temperature electron paramagnetic resonance (EPR) signals near $g = 1.94$, on reduction.[1-4] These proteins include oxidases of the xanthine type, ferredoxins, and nonheme-iron and iron flavoproteins from mitochondria and bacteria. By substitution with iron[5] and sulfur[6, 7] isotopes having nonzero nuclear spin, both elements were conclusively shown to participate in the paramagnetic center. The nuclear magnetic moments may couple to the moment of the unpaired electron of the center, producing small positive and negative incremental local fields which act to split or broaden the EPR spectrum compared to the unsubstituted cases. The effect depends, in the simplest case of isotropic hyperfine interaction, on three variables, viz., the enrichment in isotope of nonzero nuclear spin (Fe^{57} of S^{33} in the present case), the effective local field contribution or hyperfine splitting constant of each atom, and the number of atoms of each isotope involved with a single unpaired electron. If the enrichment can be estimated, trial spectra can be calculated for various hyperfine splittings and numbers of atoms. In favorable cases comparison to the observed spectrum will reveal the values of the variables that best fit hypothesis to experiment. In this way one can determine the number and kinds of atoms interacting with the unpaired electron under the assumption of predominantly isotropic hyperfine interaction.

This approach has been adopted in the present work where the exchange of Fe^{57} for Fe^{56} (and S^{33} for S^{32}) in the iron protein, putidaredoxin, of a methylene hydroxylase of *Pseudomonas putida*[8] has allowed the study of the question of how many iron (and sulfur) atoms are involved in the paramagnetic center of this material. In addition, supporting evidence was obtained from an anaerobic reductive titration of the protein.

Because of the ubiquitous distribution of the paramagnetic species exhibiting the $g = 1.94$ signal on reduction as an electron carrier,[2, 3] and because this type of EPR signal is atypical of iron compounds with well-understood structures, much discussion of this entity has taken place. Ultimately, the chemical structures and molecular orbital descriptions of these complexes must account for the optical spectra,[13] the redox potentials ranging from +220 to −430 mv,[13, 8] and the EPR spectra on reduction which feature an average g value less than the free-electron value.[2] (Occasionally this last point has not been appreciated, and model complexes[10] with EPR spectra straddling $g = 2$, in the fashion of low-spin heme complexes, are not pertinent to the $g = 1.94$ problem. Average g-values are calculated from $g^2_{ave} = {}^1/_3 (g_x^2 + g_y^2 + g_z^2) = {}^1/_3 (2g_\perp^2 + g_\parallel^2)$.

Representative values are 2.31 for a typical low-spin Fe^{III} complex, metmyoglobin azide,[22] 1.96 for the *Azotobacter* nonheme-iron protein,[5] and 1.97 for reduced nitroprusside.[11] The complex reported by Röder and Bayer[10] apparently exhibits an average g-value of 2.21, and thus does not fall in the class of complexes considered here.)

Van Voorst and Hemmerich[11] have discussed the reduced nitroprusside model $g = 1.94$ compound of Beinert *et al.*,[9] and have pointed out that a molecular orbital scheme predicting a $g = 1.94$ signal can be given for an asymmetric complex in which iron shares the unpaired electron with one of the ligands, the formal valence of the iron being undetermined. Blumberg and Peisach[12] have shown experimentally that free radical species bonded to diamagnetic ferrous iron can also give rise to the signal, the key point being that the s-character of the bond allows the large splitting observed. Brintzinger *et al.*[13] have given detailed consideration to both one- and two-iron complexes in which strong field tetrahedral symmetry leads to the observation of g values below 2. An ingenious feature of this model is that in the two-iron atom case, the oxidized complex is composed of vicinal paramagnetic low-spin iron atoms which constitute a diamagnetic or exchange-broadened entity not observable by EPR, while the addition of one electron to the complex reduces one iron atom to a diamagnetic state, whereupon the remaining paramagnetic atom gives rise to the characteristic EPR signal. This model has been criticized by Gibson and Thornley *et al.*,[14, 15] who propose an alternative based on an antiferromagnetic interaction of two high-spin ferric iron nuclei, possibly via an intervening ligand, presumably sulfur. On one-electron reduction such a complex would become paramagnetic.

On a fundamental level, the first two models are compatible with one-iron complexes, the third scheme will accommodate one- or two-iron systems, and the fourth proposal requires two-iron atoms per EPR-observable complex. Since the number of atomic participants in any of the protein cases has been heretofore unknown, we have addressed ourselves to this problem, and in this and in a succeeding paper[16] the case of putidaredoxin, an iron protein of *Pseudomonas*, is discussed from the point of view of EPR and Mössbauer spectroscopy.

Materials and Methods.—Putidaredoxin, properties and exchange of iron and acid-labile sulfur: The putidaredoxin component of the inducible methylene hydroxylase system for (+)-camphor was purified according to our published methods.[8] The samples employed contained 140–170 ngm-atoms each of iron and acid-labile sulfur/mg protein. This is equivalent to 2-gm atoms of each per mole (12,000 gm) protein. Protein estimations are based on the biuret reaction using bovine serum albumin (BSA) as a standard. Biuret readings of the enzymes were multiplied by a factor of 0.75 to relate the dry weight of acid-precipitated apoprotein and BSA. Accurate protein determinations are not easily obtained with this colored protein possessing reducing activity, especially under our present conditions using Tris buffer.

Absorption bands appear at 280, 325, 415, and 455 mμ with molar extinction coefficients near 22,500, 15,000, 10,000, and 9,600, respectively. Upon reduction with one electron per mole of protein, using dithionite, or upon enzymatic reduction,[8] the spectrum is bleached. The reduced spectrum shows a new peak at 545 mμ. The apoprotein exhibits only 280 mμ absorption (about 50% that of the native enzyme).

To prepare iron- and sulfide-free apoprotein, the enzyme and all required reagents were briefly purged with argon in a room-temperature anaerobic glove-box.[17] After 1 hr of further equilibration under argon, trichloroacetic acid (TCA), 100% w/v, was

added dropwise to a final concentration of 20%. The suspension was purged with argon for 5–10 min and the precipitate recovered by centrifugation in the anaerobic box. The white precipitate was washed with 20% TCA, collected by centrifugation, and suspended in water which was brought to pH 8.3 with Tris base.

For reconstitution, β-mercaptoethanol was added to the resultant apoprotein solution to a final concentration of 0.01 *M* followed by a sixfold molar excess each of ferrous iron and sodium sulfide. After a 20- to 30-min incubation at room temperature, the solution was removed from the anaerobic box and applied to a column of *O*-(diethylaminoethyl) cellulose (DEAE-cellulose) equilibrated with 0.05 *M* Tris-Cl and 0.01 *M* β-mercaptoethanol. The column was washed with this buffer and the red-brown protein was finally eluted with buffer to which KCl was added to a concentration of 0.5 *M*.

Typically, native putidaredoxin could be converted to the apoprotein quantitatively, and from this the holoprotein could be reconstituted in about 75% yield. The specific activity[8] of the purified reconstituted protein was usually higher than that of the starting material. The iron and sulfide contents (determined chemically or radiochemically) of the native and reconstituted materials were within 15% of the theoretical for 2-gm-atoms of element per 12,000 gm of protein, while the apoprotein had less than 1% of the iron and sulfur of the native protein.

The Fe^{57}-enriched enzyme was prepared with a nominally 90.7% enriched iron sample and the S^{33}-enriched protein was made from a nominally 48.5% enriched sulfide sample. (Both isotopes were obtained from the Oak Ridge National Laboratory.) The Fe^{57}-enriched Fe_2O_3 was dissolved in acid and reduced with dithioerythritol; the sulfide was prepared by hydrogenation of elemental enriched sulfur at 1000°C over quartz, followed by trapping the H_2S in alkali.

EPR spectroscopy: EPR spectra were obtained on a modified Varian X-band instrument with an improved low-temperature gas-flow accessory[18] and 100-kc modulation. EPR signal information was recorded with a Honeywell LAR 7400 tape recorder with evenly spaced control pulses (field markers) on an adjacent tape channel. Such a recording of an EPR spectrum of Fe^{56}-putidaredoxin could be used to simulate theoretical spectra for comparison to the observed (broadened) spectrum of the Fe^{57}-protein. This was done by superposition of several spectra of the Fe^{56}-protein. The centers of the component spectra were spaced according to the amount of hyperfine splitting desired in the composite spectrum. Also, the amplitude of each component spectrum was corrected to account for the statistical distribution of nuclear magnetic field contributions and for the enrichment in Fe^{57} (see Table 1). The component spectra were added together in a Varian time-averaging computer (C-1024) to give a simulated Fe^{57} spectrum which could be compared to the observed spectrum of Fe^{57}-putidaredoxin.

Reductive titration of putidaredoxin: The titration was carried out in an anaerobic EPR tube fitted with side arms in which a vacuum-encapsulated weighed sample of solid-diluted dithionite was held during evacuation and inert-gas flushing of the protein solution. The glass reductant capsule could then be broken to allow the reductant and protein solution to mix.[19] The reductant was standardized in analogous optical absorption cells using riboflavin 5′-phosphate (FMN) or cytochrome *c* as the oxidant. For each point in the titration after mixing of reductant and protein, the reduced protein was shaken down into the round EPR tube and scanned at *ca.* 10°C in a modified Cary 14 spectrophotometer;[20] then the protein was frozen in liquid nitrogen for EPR spectroscopy.

TABLE 1. *Splittings and intensities required for simulation of best-fit Fe^{57}-spectrum.*

Center position of component spectrum, relative to center of composite (gauss)	−14	−7	0	+7	+14
Intensities due to:					
(1) Molecules with 2 Fe^{56} nuclei	...	...	0.0036	...	...
(2) Molecules with 2 Fe^{57} nuclei	0.221	...	0.442	...	0.221
(3) Molecules with 1-Fe^{56} and 1-Fe^{57} nucleus	...	0.056	...	0.056	...
Totals:	0.221	0.056	0.446	0.056	0.221

In Figs. 1–5 the field markers (*abscissa*) are in gauss; the ordinate is an arbitrary measure of the first derivative of the EPR absorption curve. Conditions of EPR spectroscopy were: microwave power, 27 mwatt; frequency, 9,248 MHz; modulation amplitude, 5 gauss; scanning rate, 200 gauss/min; time constant, 0.5 sec; temperature, 107°K.

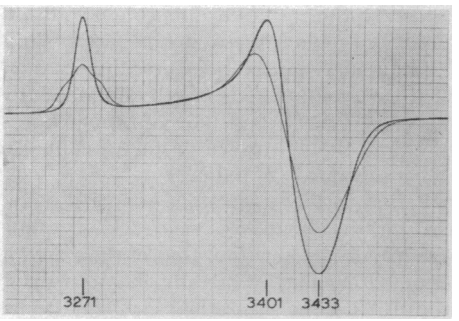

FIG. 1.—EPR spectra of reduced putidaredoxin. The spectra of the native protein and after reconstitution with Fe^{56} are superimposed and indistinguishable. The broadened spectrum is the putidaredoxin reconstituted with Fe^{57} at 90.7% enrichment. Line amplitudes are adjusted so the three curves represent equal quantities of unpaired electrons.

Results.—The EPR spectra of reduced native Fe^{56}-substituted and Fe^{57}-substituted putidaredoxin can be seen in Figure 1, which illustrates both the fact that the substitution procedure itself does not alter the signal shape or the magnitude of the broadening afforded by the Fe^{57} nucleus. Table 1 gives the values of the parameters yielding the best fit we were able to make between simulated and observed Fe^{57}-enriched putidaredoxin. The values in Table 1 correspond to 94 per cent enrichment in Fe^{57} and a 14-gauss hyperfine interaction with two Fe nuclei per molecule participating. One can see in Figure 2 the closeness of the fit between the observed and calculated Fe^{57} spectra. Figure 3 shows an enlarged view of this comparison for the $g = 2$ region of the spectrum; a correspondingly close match is found in the $g = 1.94$ region. When simulations were made on the assumption of other enrichments, splittings, or number of iron atoms, discrepancies between simulated and observed spectra arose. An example, for a (91% Fe^{57}) one-iron atom model with various hyperfine splittings, is given in Figure 4.

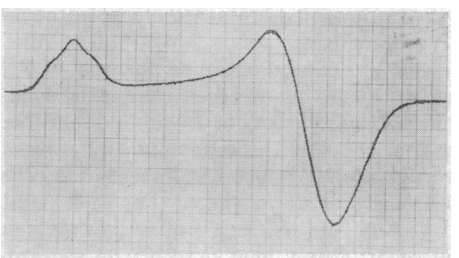

FIG. 2.—Computer simulation of EPR spectrum of Fe^{57}-substituted reduced putidaredoxin, based on the spectrum of the reduced native protein and interaction of the unpaired electron with two-iron nuclei. The Fe^{57}-enrichment was assumed to be 94% and the splitting, 14 gauss. The spectrum of the reduced protein, in which iron has been exchanged with Fe^{57}, is superimposed on the computed spectrum. The conditions of EPR spectroscopy were those of Fig. 1.

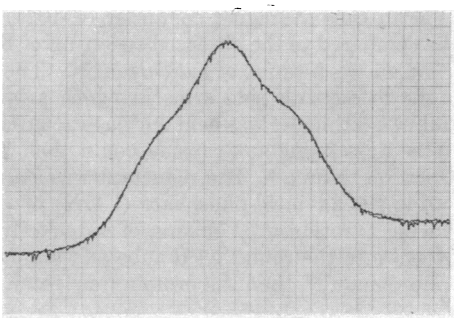

FIG. 3.—Superposition of low field ($g = 2$) portions of computer-stimulated and actual EPR spectra of reduced putidaredoxin reconstituted with Fe^{57}. Assumptions and conditions as for Fig. 1, except the scanning rate was 40 gauss/min.

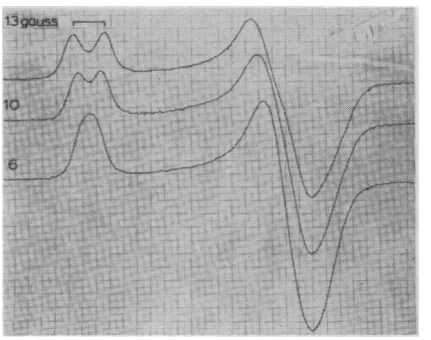

FIG. 4.—Computer simulation of EPR spectra of Fe^{57}-substituted reduced putidaredoxin based on the spectrum of the dithionite-reduced protein and interaction of the unpaired electron with one Fe^{57} nucleus. $Iron^{57}$-enrichment assumed to be 91% and the splitting 13, 10, and 6 gauss as indicated. The conditions of EPR spectroscopy for the sample of native protein were those of Fig. 1.

Fair fits to the observed spectrum could be obtained for the single-iron atom model if enrichments of less than 50 per cent and hyperfine splittings of 28 gauss were used. Therefore, a sample of the reconstituted protein (with the 90.7 per cent enriched Fe^{57}) was analyzed by the Oak Ridge Isotope Department, which reported a value of 80 ± 3 per cent Fe^{57}. With this value for the enrichment we could not simulate a spectrum fitting the observed spectrum for any assumptions about the parameters. Since the enrichment required for the one-iron model to approach a match to the observed spectra would be less than 50 per cent, and since contamination of samples with adventitious Fe^{56} is a serious problem at this level of operations—wet ashing of a putidaredoxin sample containing 100 μg Fe—we are probably justified in assuming that the percentage of Fe^{57} in active sites in the putidaredoxin molecule is closer to the 94 per cent required by the simulation results than to the mass spectral analysis. In support of this, two different Fe^{57}-reconstituted samples gave identical EPR spectra, which would be unlikely if adventitious iron contamination were influencing the EPR results.

The results of substituting the acid-labile sulfur moiety of the protein with 48.5 per cent S^{33}-sulfide can be seen in Figure 5.

When the titration of the protein was carried out with the solid-diluted dithionite method,[19] the titration curves of Figure 6 were recorded. A sharp break in the curve which depicts the yield of signal versus number of equivalents of e^- added per iron occurred at $1e^-/2Fe$, and a comparison of the double integral of the EPR signal at this point with that of a standard copper-ethylenediaminetetraacetate solution indicated that the spins observed quantitatively represented the reducing equivalents added. Figure 6 also shows the behavior of some optical spectral features during reduction. The peaks at 415 and 455 decrease in a fashion resembling the appearance of the EPR signal, the peak at 545 mμ remains relatively unchanged, and the behavior of the peak at 325 mμ (which increases sharply after 0.5 Eq/Fe

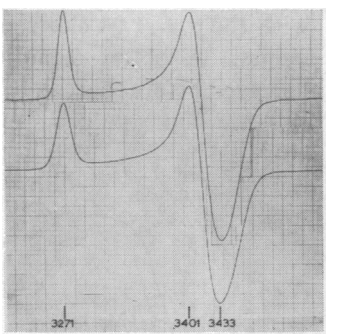

FIG. 5.—EPR spectra of reduced putidaredoxin in native form (*upper curve*) and after exchange with S^{33}-sulfide (*lower curve*). The S^{33}-enrichment was 48.5%. Curves represent equal numbers of unpaired spins; field markers in gauss. Conditions of EPR spectroscopy as in Fig. 1 except scanning rate 400 gauss/min and temperature 100°K.

is passed) gives evidence that after $^1/_2$ e$^-$/Fe is added, the additional dithionite simply remains unoxidized.

Discussion.—The procedure of Malkin and Rabinowitz[21] for exchange of iron and sulfide in ferredoxin was found applicable to putidaredoxin, but the simple acid-precipitation procedure described here worked as well or better than the older procedure and was used in the preparation of both Fe^{57}- and S^{33}-enriched materials.

The Fe^{57} enriched protein gave on reduction an EPR spectrum that could be accurately simulated with a simple isotropic hyperfine interaction model. On this basis, if the Fe^{57} enrichment were less than 50 per cent, a one-iron model would obtain, while a 94 per cent enrichment would lead to a two-iron model. The finding of 80 per cent Fe^{57} in the mass-spectral analysis supports the two-iron model, since contamination of the protein during ashing and the subsequent analytical manipulations can lower but never raise the enrichment figure. The two-iron atoms are not necessarily equivalent as consideration of the model of Gibson[14] and Thornley[15] shows.

The EPR and visible spectroscopic titrations support the idea of a two-iron unit as the electron-accepting site. The titration shows that one electron is taken up for each pair of iron atoms, that this electron is accounted for quantitatively in the EPR signal, and that those spectral features that do change on reductive titration change in concert. Of course, one might equally well suppose from the titration data alone that one of the iron atoms in the molecule is simply inert, but the isotopic substitution experiment is not in agreement with such an assumption. The substitution of S^{33}-sulfide for the S^{32}-sulfide present in the protein as isolated[8] gave a signal broadening insufficient to account for that found in the earlier growth experiments.[7] This indicates that in the protein derived from bacteria grown in the presence of S^{33}-sulfate, more sulfur nuclei interact with the $g = 1.94$ electron than are provided by sulfide in the exchange experiments. A better signal-to-noise ratio for the growth experiment must be achieved before this can be explored further.

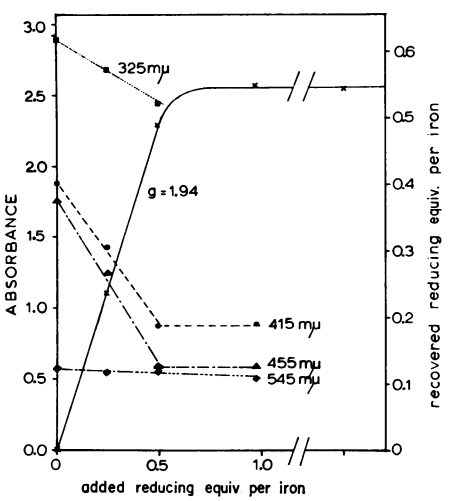

FIG. 6.—Titration of putidaredoxin with dithionite. Aliquots of solid-diluted dithionite were added to 0.200 mM (0.400 mM Fe) anaerobic putidaredoxin samples as described in the text. The left-hand scale is given in absorbance units, corrected to a 1-cm light path and for variations in protein concentrations brought on during degassing of the samples. The right-hand scale gives the unpaired spins found on comparison of the EPR signal of the samples to a standard of copper-EDTA, under the instrumental conditions of Fig. 1. The unpaired spins are given as reducing equivalents. The results are plotted as a function of the number of equivalents of dithionite added, per iron-atom-equivalent in the preparation.

Our studies so far indicate that the appearance of the paramagnetic species in putidaredoxin is due to the addition of one electron to each protein molecule, that this electron resides in a complex incorporating both iron atoms and more than one kind of sulfur group, and that the appearance of the $g = 1.94$ signal is directly correlated with absorbance changes in the visible spectrum at 415 and 455 mμ. Evidently, the strong reducing agent dithionite can only force one electron on each molecule of putidaredoxin on the basis of the behavior of the 325-mμ absorption.

These results do not allow us to choose conclusively among the physical models proposed so far,[11-15] though for this protein at least, complexes involving a single iron atom appear to be eliminated. The physical models have been very general in conception in the absence of specific information about the composition of the complex responsible for the $g = 1.94$ signal.

We feel that to supply such information, guided by the possibilities suggested by the model studies, is a task worth the efforts of those interested in the structure and function of the "nonheme-iron" proteins.

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