IDENTIFICATION OF SULFUR AS COMPONENT OF THE EPR SIGNAL AT g = 1.94 BY ISOTOPIC SUBSTITUTION*

D. V. DerVartanian, W. H. Orme-Johnson, Raymond E. Hansen and Helmut Beinert
Institute for Enzyme Research, University of Wisconsin, Madison

R. L. Tsai, J.C.M. Tsibris, R. C. Bartholomaus, and I. C. Gunsalus
Biochemistry Division, Department of Chemistry and Chemical Engineering, University of Illinois, Urbana

Received January 27, 1967

Shethna et al. (1) showed by substitution of Fe^{57} for Fe^{56} in a non heme iron protein from Azotobacter vinelandii, that the EPR signal at g = 1.94, observed on reduction of this type of protein, was due to a structure containing iron. Evidence from chemical analyses indicated that this and related proteins contained - in a particularly labile form - sulfur which was essential for their integrity and function (2). It was, therefore, generally assumed that the structure responsible for the EPR signal, which is unusual for iron complexes, might involve sulfur as a ligand of iron. It was merely a matter of availability of the proper sulfur isotope in sufficient enrichment (S^{33}, nuclear spin 3/2, magnetic moment 0.64) to attack the question in a fashion analogous to that of the involvement of iron in the signal. Preliminary results have been published by Hollocher et al (3) on whole cells of azotobacter, which indicate that the splitting by S^{33}

*This work was supported in part by grants from the National Institutes of Health, USPHS, (GM 12394, AM 00562), a Research Career Program Award (GM-KG-18,442) to H.B., a postdoctoral fellowship to W.H.O.-J., (2-F2-GM 10,236), and a grant from the National Science Foundation G24037.

is indeed of sufficient magnitude to be observable. In view of the importance of the question, as to whether sulfur is indeed represented in the g = 1.94 signal, or in other words, whether an electron taken up by the iron complex on reduction is shared by sulfur and iron atoms, we considered it important to obtain a definitive answer.

When S^{33} of 48.5% enrichment became available, the isotope was incorporated into the iron proteins of two different bacteria: Azotobacter vinelandii and Pseudomonas putida. The EPR signals of the purified reduced proteins were compared to the corresponding signals obtained with the proteins from S^{32} grown cells, and with calculated spectra. The effects observed on both components of the EPR signal (g = 1.9 and g = 2; see Figs. 1-5) show unequivocally that sulfur does contribute to the signal in the two proteins.

S^{33} of 48.5% enrichment was purchased from Oak Ridge National Laboratory and converted to Na_2SO_4 by oxidation with fuming nitric acid. Conditions for growth on a minimum of sulfur were established. For both bacteria approximately 3mg of sulfur per liter of growth medium were needed. Manganese was omitted from the medium for pseudomonas because of interference in EPR spectroscopy. Separate lots of each organism were grown, several on S^{32} and two on S^{33}, and were worked up separately. The iron proteins were purified on a small scale according to methods described previously (4-6). The iron protein from azotobacter was carried through the ammonium sulfate precipitation step and that from pseudomonas through the DEAE eluate step followed by precipitation with ammonium sulfate at 75% saturation. The proteins were reduced by addition of a moderate excess of dithionite and the EPR spectra were superimposed on the spectra of proteins obtained from cells grown on S^{32} in a manner analogous to the S^{33} cells.

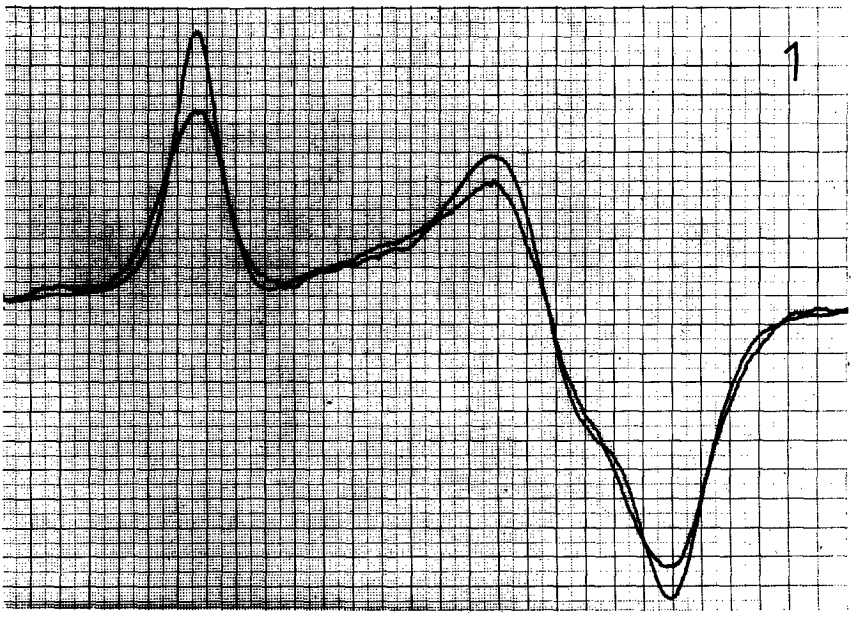

Fig. 1. Photographic reproductions of original EPR spectra of iron protein purified from S^{32} and S^{33} grown cells of azotobacter. The signal with the higher amplitude is that observed with protein from S^{32} grown cells. For comparison the amplitudes were adjusted so that the total integrated intensities of both signals match. Approximately 30 mg of protein were dissolved in 0.25 ml of 0.1 M potassium phosphate of pH 7.4 and reduced with dithionite. The microwave frequency was 9,248 MHz/sec, the power 23 mw, modulation amplitude 5 gauss, scanning rate 50 gauss per minute, time constant 2.5 sec, and the temperature 97°K.

The previous experiments on Fe^{57}-grown cells (1) can serve as a guide to the spectral changes which may be expected: a general broadening, particularly well expressed in the g_z ($g = 2.01$) component of the signal, and abolishment of the separation of g_x and g_y (shoulder on main peak at $g = 1.94$). The latter effect is particularly significant and diagnostic (see Fig. 5 and ref. 1, Figs. 1-3) but does only occur, when g_x and g_y differ sufficiently as for the azotobacter protein. According to calculation and computer simulation (cf. Fig. 2 and ref. 1, Fig. 3) it is an obligatory sequel of hyperfine splitting plus superposition of the S^{33} (or Fe^{57})

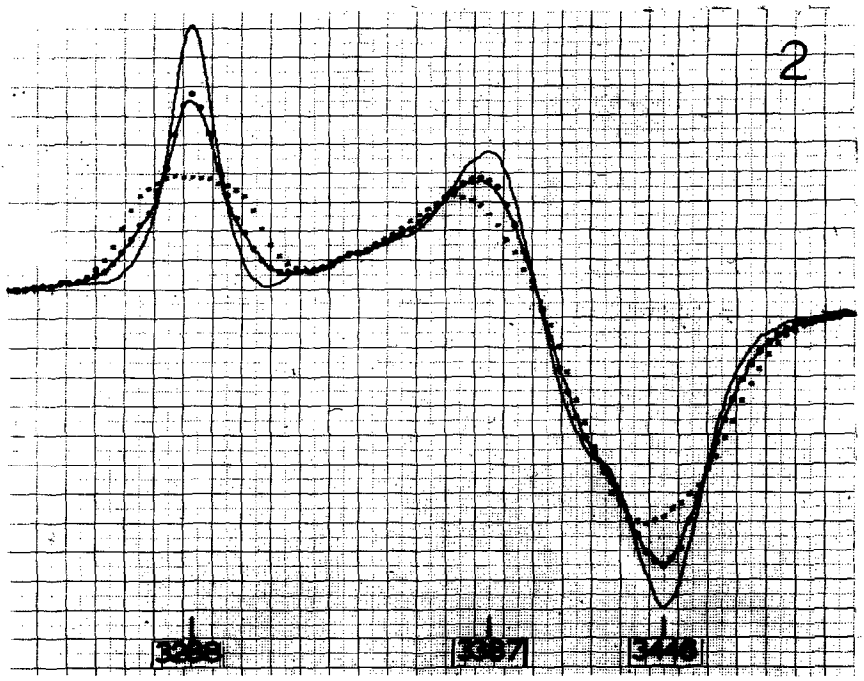

Fig. 2. EPR spectrum of iron protein from S^{32} grown cells of azotobacter (larger amplitude) superimposed on computer-simulated spectrum for protein labeled with S^{33} (smaller amplitude) with an assumed enrichment of 45% S^{33} and a splitting of 12 gauss. The dots indicate the manually calculated amplitudes for the same conditions and the crosses those for 100% S^{33} and 12 gauss splitting. The values along the abscissa indicate the field in gauss.

---

spectrum on the S^{32} or (Fe^{56}) spectrum. The apparent changes at g_z are not as reliable an indication of the isotope effect, since at this g value, where most EPR signals are found, particularly free radical signals, interference can be expected and may indeed be serious. At the relatively high modulation and power settings required for observation of the EPR spectra of the small quantities of labeled iron proteins available, the radical signals will be partly saturated and may be distorted by modulation (7). It is pertinent here that the flavoprotein which accompanies the iron protein from azotobacter (4) is particularly prone to distortion (8). This may result in mis-

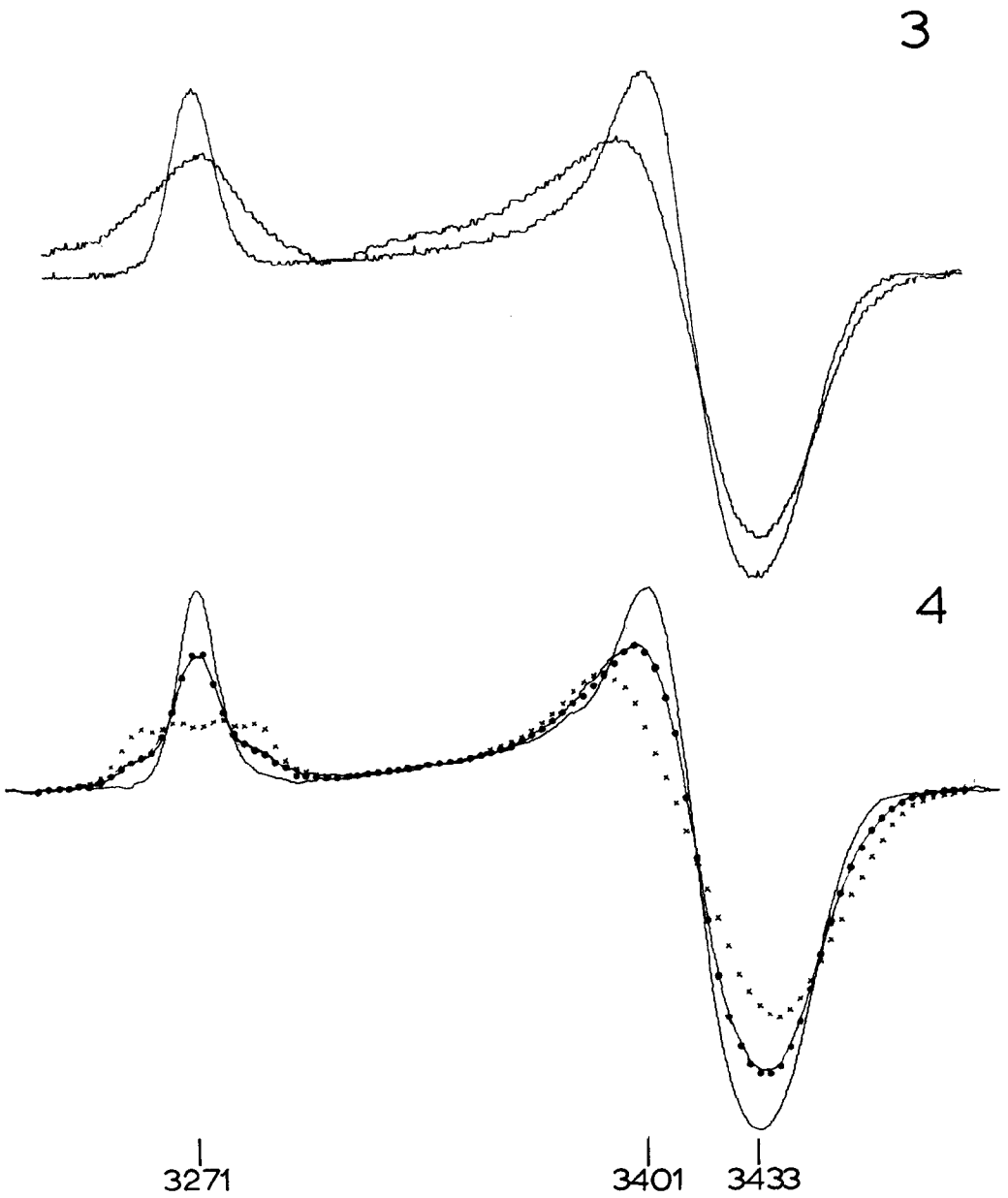

Fig. 3. Photographic reproductions of original EPR spectra of iron protein from pseudomonas analogous to Fig. 1. Approximately 3 mg of protein were dissolved and reduced as in Fig. 1 in the presence of 10 mM mercaptoethanol. 16 spectra were accumulated in the C-1024 computer before display. The microwave frequency was 9,248 MHz/sec, the power 91 mw, modulation amplitude 7 gauss, scanning rate 500 gauss per minute, time constant 0.25 sec, and the temperature 85°K. The g = 1.94 signal from this protein is not saturated under these conditions.

Fig. 4. EPR spectrum of iron protein from S^{32} grown cells of pseudomonas (larger amplitude) superimposed on computer-simulated spectrum for protein labeled with S^{33} (smaller amplitude), prepared and presented analogous to Fig. 2. The conditions of EPR spectroscopy for recording the S^{32} spectrum were those of Fig. 1.

---

interpretations of the behavior of the g_z component on isotopic substitution. Although it may appear that in the spectra shown here such signals are absent, minor interference from small superimposed signals in this spectral region is nevertheless evident from the deviations from the calculated spectra. It is therefore important that the effect of the isotope is also clearly observed at the main component where interference from radical signals is excluded.

The calculations of the S^{33} spectra were based on hyperfine interaction of one sulfur atom, since the ratio of labile sulfide to iron ≤ 1.

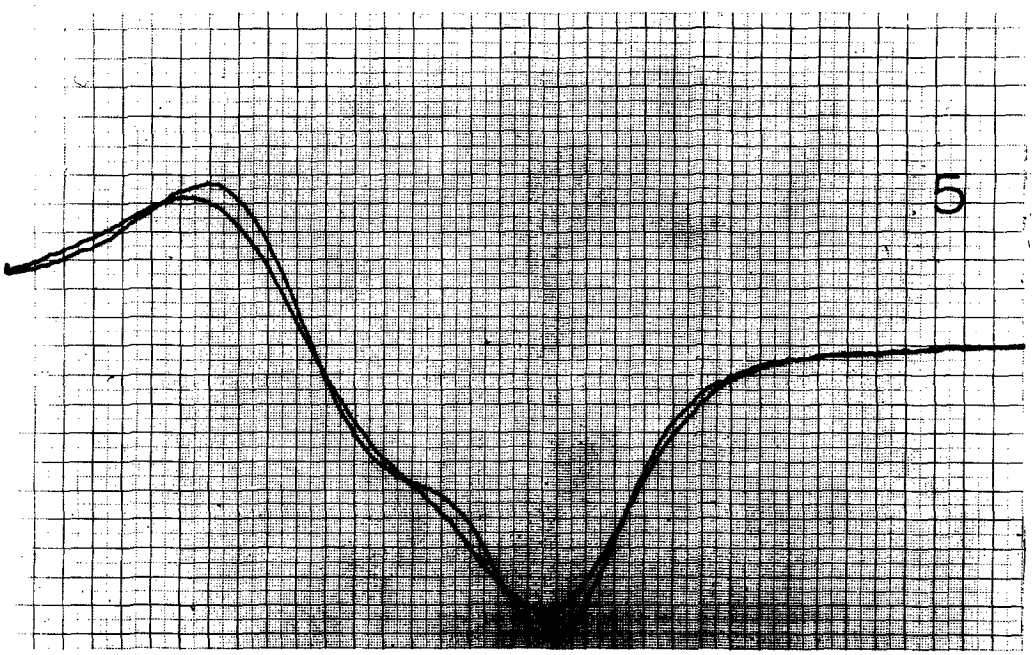

Fig. 5. Expanded portion of EPR spectrum of Fig. 1 demonstrating the effect of hyperfine splitting by S^{33} on the main peak of the signal, as shown in the calculated spectra of Fig. 2. The conditions were those of Fig. 1, except for a 2 fold expansion of the scale on the abscissa.

The intensity observed at points along the abscissa of the S^{32} curves was distributed over four lines with the assumed separation. The S^{33} spectra so obtained were then superimposed on an S^{32} spectrum with the relative intensities adjusted according to the assumed enrichment. Only isotropic hyperfine interaction was considered and no correction was made for changes in transition probabilities. Simulated spectra were obtained in an analogous fashion by the use of the Varian C-1024 time averaging computer. S^{32} curves were accumulated in the computer, one each centered at the assumed field position of each hyperfine line and one at the center. The proper enrichment was again obtained by adjusting the relative amplitudes.

In these calculations and simulations assumptions as to hyperfine splitting and final enrichment had to be made. Since a 1% inoculum of cells grown on minimal sulfur medium was used, it is unlikely that substantial dilution of an isotope such as sulfur could occur. A final enrichment of 45% was therefore assumed. With assumed enrichments between 40 and 50%, the best fit of the computed curves to the original curves of the proteins from either source was obtained with a splitting of 12 gauss between individual lines of S^{33}. On the basis of a comparison with calculated and simulated curves, we think that only one sulfur atom may be involved in the hyperfine interaction and that anisotropic hyperfine interaction makes no major contribution. The actual value of the splitting is estimated to lie within 15% of this value. It is interesting to note that Pettersson and Vänngård found splittings of 11.2 and 11.9 gauss by S^{33} in Cu(II) and Ag(II) dithiocarbamates, respectively (9).

REFERENCES

1. Y.I. Shethna, P.W. Wilson, R.E. Hansen, and H. Beinert, Proc. Natl. Acad. Sci. 52, 1263 (1964).
2. Non-Heme Iron Proteins: Role in Energy Conversion, A. San Pietro, ed., Antioch Press, Yellow Springs (1965).
3. T.C. Hollocher, F. Solomon and T.E. Ragland, J. Biol. Chem. 241, 3452 (1966

4. H. Beinert, Y.I. Shethna, and P.W. Wilson, Biochem. Biophys. Acta, 113, 225 (1966).
5. D.W. Cushman, R.L. Tsai, and I.C. Gunsalus, Biochem. Biophys. Res. Commun., 26, 577 (1967).
6. J. Hedegaard and I.C. Gunsalus, J. Biol. Chem. 240, 4038 (1965).
7. K. Halbach, Helv. Phys. Acta, 27, 259 (1954).
8. H. Beinert, and W.H. Orme-Johnson, "Magnetic Resonance in Biological Systems", A.Ehrenberg, B.G. Malmström, and T. Vänngård, eds., Pergamon Press, London, in press.
9. R. Pettersson and T. Vänngård, Arkiv for Kemi, 17, 249 (1961).