STUDY OF THE ADRENAL NON-HEME IRON PROTEIN (ADRENODOXIN*) BY ELECTRON SPIN RESONANCE

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Received June 2, 1966

In a previous communication (Suzuki and Kimura, 1965), adrenodoxin, a ferredoxin-like non-heme iron protein, was isolated in a homogeneous state from pig adrenals. Adrenodoxin was shown to be one of the redox components of the adrenal steroid hydroxylase complex, with a molecular weight of about 22,000 and two atoms of iron and two moles of labile sulfide per mole of protein****. As has been suggested (Kimura and Suzuki, 1965), the iron atoms which are coordinated with the protein moiety, play a key role of its catalytic function.

The present work will provide imformation on the function of adrenodoxin with respect to the state of the iron, as studied by electron spin resonance.

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* We proposed the trivial name, "adrenodoxin", for the non-heme iron protein isolated from adrenal mitochondria, in order to avoid possible confusion among non-heme iron proteins which may exist in the adrenals.

**** Although an earlier study (Suzuki and Kimura, 1965) had indicated one atom of iron per molecule of adrenodoxin, precautions to prevent loss of iron during the preparation procedures showed the protein to contain two atoms of iron per molecule.

This study is in part supported by a grant from National Institutes of Health, USPHS, (AM 09243) to T.K. and by funds from the Ministry of Education in Japan.

Adrenodoxin and a NADPH dehydrogenase (adrenodoxin reductase) were prepared as previously described with minor modifications (Suzuki and Kimura, 1965)(Kimura and Suzuki, 1965). Labile sulfide was determined by the method of Foge and Popowsky (1949) with slight modifications: The titer of the sodium sulfide standard was determined iodometrically. Iron was determined by the method of o-phenanthroline, as adopted by Massey (1957). Protein concentrations were determined by the biuret method. Purified adrenodoxin preparations used throughout this investigation were about 50% pure, as judged by the measurement of non-heme iron content on the assumption that the purest protein has 103 mμatoms of iron per mg of the protein. At this stage of purity, there is no flavin and no heme to be detected by the fluorometric or spectrophotometric method. Further, any significant contamination of Ni, Zn, Cu, Mn and Mo in the preparation could not detect by the determination of the method of atomic absorption (Willis, 1963). Electron spin resonance was performed at the liquid nitrogen temperature with the Varian V-4500 spectrometer with 100 kc magnetic field modulation. ESR signal was plotted as X-axis and output voltage of the Hall effect element, which is porportional to magnetic strength, was plotted as Y-axis of X-Y recorder.

Iron Valence in Oxidized Adrenodoxin as Measured by Chemical Method:

As stated in the previous report (Kimura and Suzuki, 1965), when adrenodoxin was reduced either by NADPH and adrenodoxin reductase or by dithionite, bleaching throughout the visible color was prominent.

The chemical determination of the ferrous iron content in adrenodoxin was performed by colorimetric measurements of the ferrous o-phenanthroline complex with or without blocking the reducing groups in adrenodoxin. Table I shows that in the presence of an excess of PCMB all the iron could be extracted by 5% trichloroacetic acid as ferric ions, as indicated by the fact that the ferrous complex was formed only on addition of ascorbate as a reductant. Therefore, it is concluded that all the iron in

untreated oxidized adrenodoxin is in the ferric ion. Of interest, some 50% of iron atoms could be removed as ferric ions even without the presence of mercurial.

Electron Spin Resonance Spectrum of Reduced Adrenodoxin:

Upon reduction of adrenodoxin in the presence of NADPH and adrenodoxin reductase a prominent signal appeared. The ESR spectrum (first derivative curve) of the enzymatically reduced form is given in Fig.1. The g-values of this signal were calculated by Kneubühl's method (1960) as $g_{\perp}$ ($g_1 \approx g_2$) = 1.94 and $g_{\parallel}$ (g_3) = 2.01. When adrenodoxin was reoxidized by aeration, this signal disappeared. After calculation of the signal intensities a strong

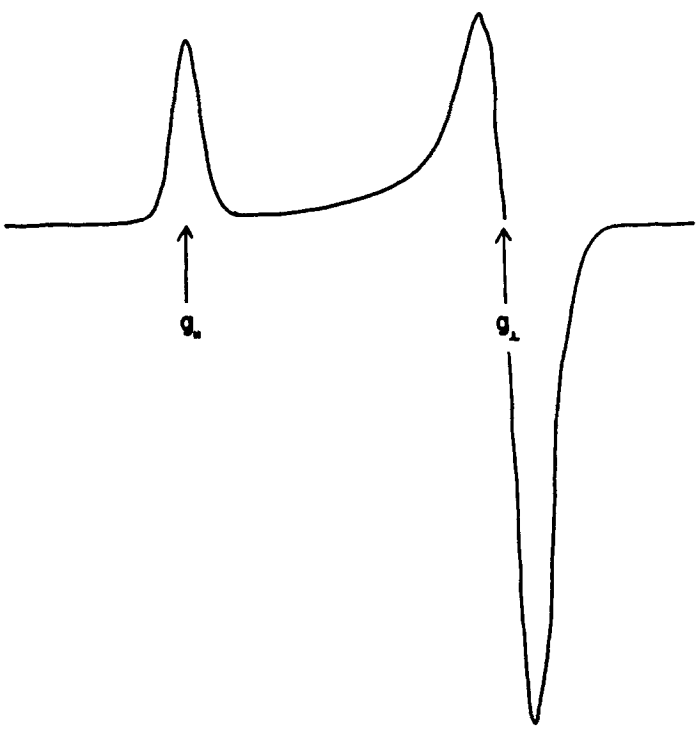

Fig.1, Electron Spin Resonance Spectrum of Reduced Adrenodoxin

The reaction mixture contained: 8 μmoles of tris buffer (pH 7.4), 0.75 μmoles of NADPH, 475 μatoms of iron in 11 mg of protein as adrenodoxin and 159 μg of protein of adrenodoxin reductase in 0.40 ml in an anaerobic cell. After the reaction mixture was incubated at 30°C for 30 minutes, the ESR spectrum was recorded at 99°K with a Varian V-4500 spectrometer set a modulation amplitude of 3.2 gauss at microwave power attenuation of 10 db.

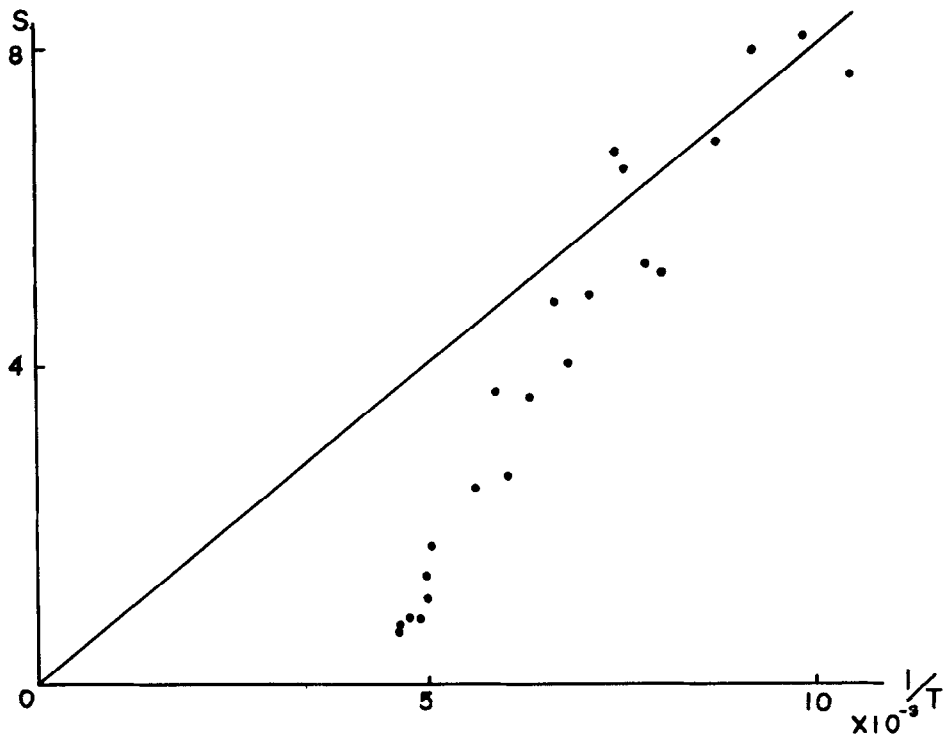

Fig.2, Dependence of ESR Signal Intensity on Temperature

The experimental conditions were same as described in Fig.1, except temperature. The signal intesities were calculated from double integration of the first derivative curve, refered to a standard solution of 1 mM $CuSO_4$.

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dependence on temperature was observed, as indicated in Fig.2. Also, Fig.3 illustrates a saturation experiment of the signal by microwave power.

A quantitative evaluation of the spectrum of reduced adrenodoxin was performed by double integration of the ESR derivative curve, and by calibration of saturation with microwave power: For a standard reference 1 mM $CuSO_4$ solution was used. The value derived from an experiment with enzymatic reduction could explained about 50% of the iron content found by the chemical method. The identical spectrum was observed upon reduction by dithionite.

The behaviour of the signal observed in reduced adrenodoxin is quite similar to that found in reduced preparations from submitochondrial

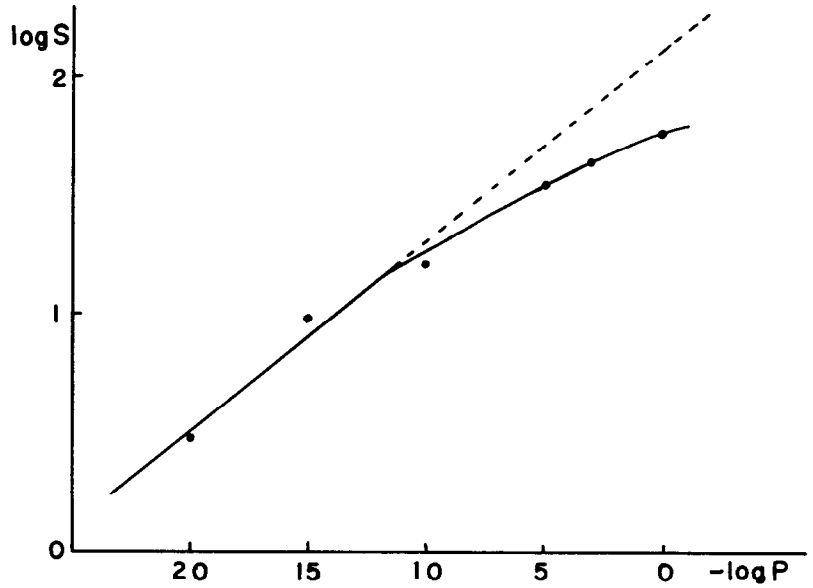

Fig.3, Saturation Experiment of Microwave Power

The experimental conditions were same as described in Fig.1, except microwave power attenuations. S: signal intesity, P: microwave power attenuation.

particles by Beinert and Sands (1960). Furthermore, similar signals have been reported in metal flavoproteins and some of the non-heme iron proteins from microorganisms. It is important to note that ferredoxin and Chromatium ferredoxin had not been observed to show a distinct signal at $g \approx 2.0$ upon reduction at 95°K, until Palmer and Sands (1966) found the signal at 40°K. Their observations showed that spinach ferredoxin has the parameters at $g_1 = 1.89$, $g_2 = 1.96$ and $g_3 = 2.04$ and attempt to produce the ESR signal by enzymatic reduction was not successful, owing to the unfavorable oxidation-reduction potentials. Since adrenodoxin has a high oxidation-reduction potential of about 150 mV (E_o'), it was easy to produce the ESR signal at $g \approx 2.0$ region upon the enzymatic reduction. However, it is suggested that there is no fundamental difference between adrenodoxin and the so-called $g = 1.94$ iron proteins. Furthermore, the fact that about half of the iron content in adrenodoxin

can be found as species which exhibit the ESR signal and which can be reduced by the intramolecular reductant seems to be pertinent to assume that each of two iron atoms would not be equivalent.

In addendum, Omura and his colleagues (1965) also isolated a non-heme iron protein from bovine adrenals and observed the reduction of the protein by NADPH and the reductase. They also briefly noted the ESR signal at g=1.94 upon reduction with dithionite.

Table I, Ferrous Iron Content in Adrenodoxin with or without Blocking Reducing Groups

Exp. No.	Reductant Added	Iron Content	Total Iron in Ferrous State
		μatoms/mg protein	%
I	with ascorbate	35.4	100
	without ascorbate	0.0	0
II	with ascorbate	39.9	100
	without ascorbate	21.6	54

The chemical determinations were performed as follows: In Experiment I, PCMB (400 μmoles) in 0.40 ml was added to adrenodoxin (100 μatoms of iron). To this solution, 1.0 ml of 15 % trichloroacetic acid was added and the contents were well mixed. After centrifugation, the supernatant solution was determined by the method of o-phenanthroline with or without the presence of ascorbate. In Experiment II, PCMB was not added.

Table II, Iron and Labile Sulfide Content in Adrenodoxin

Exp.No.	Iron[a)]	Iron[b)]	Labile Sulfide
I	41.5	19.3	38.7
II	38.4	19.8	34.6

Values were expressed by μatoms of iron or μmoles of hydrogen sulfide/mg of protein in biuret basis.
a) was determined by chemical analysis as total iron and b) was calculated by ESR analysis. In Experiment I, adrenodoxin was reduced reduced enzymatically in an anaerobic cell and in Experiment II, it was reduced chemically by dithionite.

Acknowledgement:

The authors wish to thank Mr.M.Tsuchiya and Miss G.K.Kabayashi for their excellent technical assistance.

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