Studies on Adrenal Steroid Hydroxylases

THE MAGNETIC SUSCEPTIBILITY OF OXIDIZED AND REDUCED ADRENAL IRON-SULFUR PROTEIN (ADRENODOXIN)*

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SUMMARY

The magnetic susceptibility of adrenal iron-sulfur protein (adrenodoxin) in the oxidized and reduced states was measured over the temperature range 4°-260°K. The oxidized protein was diamagnetic in the entire temperature range, and the reduced protein exhibited a paramagnetism without any deviation from Curie's law (S = 1/2). None of the theoretical curves based on exchange interaction energy between 10°K and 500°K fit the experimental curve.

Adrenal iron-sulfur protein (adrenodoxin), which serves as an oxidation-reduction intermediate in the NADPH-dependent steroid hydroxylation reactions of adrenal mitochondria, has 2 g atoms of iron and 2 moles of labile sulfur per mole of protein (1). The reduced protein exhibits a pronounced axially symmetrical electron paramagnetic resonance at $g_\parallel = g_\perp = 1.94$ and $g_\perp = 2.01$, whereas no signal is detected in the oxidized protein (2). Neither the valence nor the spin state of the iron atom present in the protein has been unambiguously defined.

The present study deals with the measurement of the magnetic susceptibility of adrenal iron-sulfur protein in both oxidized and reduced forms, in order to gain some insight into the oxidation-reduction mechanism of the protein. In particular, it is of interest to see whether or not there is a magnetic interaction between the 2 iron atoms present in this protein. For this purpose, the susceptibility measurements were carried out by changing the temperature from 4°K to 260°K, at which a deviation from simple Curie's law might occur if there is a magnetic interaction between 2 iron atoms.

During the course of this investigation, Moss, Petering, and Palmer (3) reported magnetic susceptibility measurements on spinach and parsley ferredoxins, indicating that the oxidized proteins are diamagnetic and the reduced proteins are paramagnetic. However, their data were mostly obtained around liquid helium temperature.

METHODS AND MATERIALS

The magnetic susceptibility measurements were performed by the use of two automatic balances, depending on the temperature region measured. Between 4.2°K and 100°K, a magnetometer of the chemical balance type with a sensitivity of about $10^{-2}$ dyne was used (4). In order to measure the magnetic susceptibility as a function of $1/T$, a high accuracy of temperature measurement is required at low temperature. For this purpose, a carbon resistor was inserted into the sample compartment which was lined with a thin copper plate (4). At temperatures above the boiling point of liquid nitrogen, a torsion type balance was used as described elsewhere (5).

Iron was determined by the o-phenanthroline method as described previously (6), and protein concentration was determined either by the biuret reaction or by the use of the molar extinction coefficient of 9800 at 414 mp. At the end of the magnetic susceptibility measurements, the optical absorption spectrum of the sample was examined, indicating a less than 5% change in the spectrum during the course of the experiments.

Bovine adrenal iron-sulfur protein was prepared by the simplified method as reported elsewhere (1). Starting from 5 kg of bovine adrenals, 472 mg of protein were isolated in a pure form. The ratio of Ad14 to Az5 of the preparation used in these experiments was 0.76 and the iron content was 1.9 g atoms per mole of protein. The molecular weight was assumed to be 12,000. Reduced adrenal iron-sulfur protein was prepared by enzymatic reduction in the presence of excess amounts of NADPH and a catalytic amount of the diaphorase (adrenodoxin reductase) was prepared from adrenals (6). The full reduction of the protein was confirmed by measuring the decrease of the optical absorbance at 414 mp.

RESULTS

Figs. 1 and 2 show the magnetic susceptibility measurements of the oxidized adrenal iron-sulfur protein in the ranges 77-260°K and 4-77°K, respectively. These results show clearly that the oxidized protein exhibits a temperature-independent susceptibility. A slight deviation from the theoretical curve for $S = 0$ which appears in the higher temperature region may be due to

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The effect of temperature on the molar magnetic susceptibility of oxidized adrenal iron-sulfur protein in the range 77-260°K. The concentration of adrenal iron-sulfur protein was $8.33 \times 10^{-3}$ M in 0.01 M phosphate buffer, pH 7.4. The points represent the experimentally obtained values, and the solid curves are theoretical ones with assumptions as described in the text. The concentrations were carried out by the use of the equation:

$$X_m = \frac{2N_0\mu_B^2\mu}{kT} \frac{\exp\left(-J/kT\right)}{1 + 3\exp\left(-J/kT\right)}$$

where $X_m$ is molar susceptibility, $\mu_B$ is Bohr magneton, $N_0$ is Avogadro's number, $\mu$ is splitting factor, $k$ is Boltzmann's constant, and $J$ is exchange energy, as defined by $-JS_1S_2$.

Experimental errors (Fig. 1). Although the absolute values obtained for the magnetic susceptibility may be subject to some uncertainty, the slope of the line ($X_m$ against $1/T$) is very accurate. A slight dependence on temperature observed in Fig. 2 may be due to paramagnetic impurities such as oxygen which condense on the sample cell and magnetometer. Preliminary experiments indicate that the oxidized protein also displays a temperature-independent susceptibility up to 900°K. Therefore, we conclude that the oxidized protein shows diamagnetism in the entire region of temperature.

This result is consistent with the lack of electron paramagnetic resonance of the oxidized protein. The same conclusion has been reported in bacterial ferredoxin by Druskeit, Gersonde, and Netter (7) and Blomstrom et al. (8), and in plant ferredoxins by Moss et al. (3).

As postulated by Gibson et al. (9), a possible explanation for the diamagnetism is antiferromagnetic exchange interaction between 2 iron atoms in the same molecule. This idea is later supported by Moss et al. (3), who interpreted the diamagnetism as antiferromagnetic exchange coupling between two high spin Fe(III) atoms. In Figs. 1 and 2, the theoretical curves (solid lines) are based on the following assumptions: (a) the lowest energy level is $S = 0$ and the first excited state is $S = 1$, and (b) the energy separation between these levels is assumed to be between 10°K and 500°K (as shown by the theoretical curve) in terms of strength of the exchange interaction energy ($J/k$).

The present experimental data suggest that there is no increase of magnetic susceptibility due to thermal excitation of the triplet state. Fig. 3 illustrates the magnetic susceptibility of the enzymatically reduced adrenal iron-sulfur protein in the range 77-260°K. This result indicates that the reduced form is paramagnetic. The experimental values are in good agreement with those of theory ($S = 1/2$). A separate experiment done in the range 4-77°K was consistent with the result shown in Fig. 3. Thus, the reduced protein has 1 unpaired electron per molecule of protein. This implication is compatible with our previous evaluation of the signal intensity of the electron paramagnetic resonance at $g = 1.94$; the signal intensity corresponds to half of the total iron present in the protein (2). Moss et al. (3) also found $S = 1/2$ in the reduced ferredoxins. Our results indicate that the magnetic susceptibility of reduced adrenal iron-sulfur protein does not deviate from Curie's law in the temperature region between 4°K and 260°K.
DISCUSSION

Many models for the electronic state of iron in the oxidized and reduced iron-sulfur proteins have been proposed. A model must be consistent with the following important aspects of the magnetic properties: (a) diamagnetism in the oxidized form, (b) paramagnetism \((S = 1/2)\) in the reduced form, giving rise to an electron paramagnetic resonance signal at \(g = 1.94\), and (c) the interaction of a single unpaired electron with both iron atoms in the reduced state (10).

Gibson et al. (9) proposed a model with an antiferromagnetic exchange interaction; the spins in the oxidized state are strongly antiferromagnetically coupled to give the \(S = 0\) state.

\[
\begin{array}{ccc}
\text{Oxidized} & \text{Reduced} \\
\text{Fe(III)}-\text{Fe(III)} & e & \text{Fe(III)}-\text{Fe(II)} \\
S = 0 & \rightarrow & S = 1/2
\end{array}
\]

Recently, Johnson et al. (11) postulated a model with low spin Fe(II); this is the simpler possibility as it does not require antiferromagnetic exchange coupling between the iron ions in the oxidized form.

\[
\begin{array}{ccc}
\text{Oxidized} & \text{Reduced} \\
\text{Fe(II)} & \text{Fe(II)} & e \\
\text{Low spin} & \text{low spin} & \text{low spin} \\
S = 0 & \rightarrow & S = 1/2
\end{array}
\]

Furthermore, they suggested that, with a large delocalization of electrons around the sulfur, the reduced state is electronically equal to:

\[
\begin{array}{ccc}
\text{Fe(III)} & \text{(2RS)} & \text{Fe(II)} \\
\text{Low spin} & \text{low spin} & \text{low spin} \\
S = 1/2
\end{array}
\]

It is important to compare these magnetic susceptibility data with those obtained from Mössbauer spectroscopy of ferredoxins from the following sources: *Clostridium pasteurianum* (8), *spinach* (11), (12), *Chromatium* (12), *Euglena* (11), and *Pseudomonas putida* (13). In these cases, the small isomer shift in the oxidized protein is thought to be due to a low spin Fe(II) complex (11). Actually, this shift could be due to Fe(III), high or low spin. Moreover, it should be mentioned here that it is also not far from that of model Fe(IV) compounds such as strontium ferrate(IV) (14, 15). These authors stated that the reduced electron density in the Fe(IV) compounds can be explained in terms of a covalent contribution to the 3d electronic levels which results in an increase in the screening of the \(e\)-electron (16). The isomer shift in the Fe(IV) compounds is also much less negative than that predicted by elementary theoretical calculations based on the purely ionic 3d electronic configuration (17). Both reduced putidaredoxin (13) and *Euglena* ferredoxin (11) give spectra in which 2 iron ions appear identical and are not obviously interpretable as low spin Fe(II) ions, because of the presence of the magnetic hyperfine splitting.

In the reduced form, an unpaired electron interacts with both iron ions as postulated by Beinert and Orme-Johnson (10) from their observations on \(^{57}\)Fe nuclear electron spin interaction. The observed three-line hyperfine splitting from \(^{57}\)Fe \((I = 1/2)\) requires that the unpaired electron interact approximately equally with each of the 2 iron nuclei in the protein, implying a binuclear iron complex with strong coupling between the 2 iron atoms.

It has been known that antiferromagnetic exchange coupling energies of some iron compounds are considerably lower than room temperature: FeO (f. c. c.) 183°K, FeF₂ (b. c. c.) 78.4°K; FeCl₂ (h. c. p.) 23.5°K; FeO₁₀₂·₄·₅·₉ (perovskitelike), 134°K. However, there are at least two examples of \(S = 1/2\) systems with sulfur or oxygen ligands. Coucouvanis, Lippard, and Zubieta (18) characterized the dimeric Fe(III) compound [Fe(SC₂H₅)₉]₄ (SC₂H₅)₂ as a possible model for iron-sulfur proteins which is diamagnetic at room temperature. Another example is the iron-hydroxy dimer formed in ferric perchlorate solution which appears to be diamagnetic at room temperature (19).

These systems imply that very strong coupling of metal ions in binuclear complexes are possible. In consequence, the results presented here appear to be suggestive of a very high lower limit of exchange coupling energy for a Fe(III) binuclear complex in adrenal iron-sulfur protein.

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Addendum—In a recent publication, Poe et al. (20) reported that the magnetic susceptibility of oxidized ferredoxin from *C. pasteurianum*, which contains 6 to 8 iron atoms per molecule, increases with temperature in the range from 270° to 388°K. This suggests extensive antiferromagnetic exchange coupling between the iron atoms. The data presented in the present paper covering the range from 4° to 260°K on adrenodoxin, which contains 2 iron atoms per molecule, neither directly support nor contradict the hypothesis of antiferromagnetic coupling.

REFERENCES


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