The Magnetic Susceptibility of Oxidized and Reduced Ferredoxins from Spinach and Parsley and the High Potential Protein from Chromatium*

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SUMMARY

We have measured the magnetic susceptibility of the iron-sulfur proteins, ferredoxin from both spinach and parsley, and high potential protein from Chromatium in the oxidized and reduced state.

Over the temperature range 1.4-201° K the oxidized plant proteins are either totally diamagnetic or exhibit trace paramagnetism. The reduced proteins are strongly paramagnetic with a susceptibility appropriate for a single unpaired electron per molecule of protein.

The protein from Chromatium is strongly paramagnetic in the oxidized state, again with 1 unpaired electron per molecule of protein. The reduced protein is essentially diamagnetic.

We have previously reported that spinach ferredoxin does not exhibit any electron paramagnetic resonance in the oxidized state, but, on reduction, either chemically or enzymically, an intense resonance of the \( g = 1.94 \) type is obtained (1). The signal is extremely temperature-sensitive and well resolved spectra can only be obtained below 40° K. The origins of this spectrum are currently the object of much investigation and speculation (2, 3), but the appearance of the electron paramagnetic resonance signal in the reduced form has suggested that the oxidized protein is diamagnetic and that the reduced protein has an effective spin of one-half in the ground state. Ehrenberg² has performed some preliminary room temperature susceptibility measurements which support this idea, and in this paper we report some low temperature magnetic susceptibility data on spinach and parsley ferredoxin which clearly show that this tentative assignment of spin states is correct.

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§ Career Development Awardee of the National Institutes of Health.

² A. Ehrenberg, personal communication.

EXPERIMENTAL PROCEDURE

Spinach and parsley ferredoxin were prepared by the method of Kereztes-Nagy and Margoliash (4), slightly modified to permit working with large quantities of plant material. The critical purity ratios \( A_{480}/A_{280} \) of the preparations used in these experiments were 0.47 and 0.58 for spinach and parsley, respectively. The proteins were concentrated to approximately 3 mM by placing them in an 18/32 dialysis tubing maintained inflated by a section of glass tubing, and then submerging the dialysis sac vertically in dry Bio-Gel P-300. The concentrated protein was subsequently dialyzed against 0.15 M Tris-chloride, pH 7.9, at 5°, containing 1 M NaCl. Protein concentration was determined with the values of 9,400 and 10,100 for the molar absorbancy at 420 nm for the spinach and parsley ferredoxins, respectively.

Reduced high potential protein was prepared from Chromatium strain D by the method of Bartsch (6). It had a critical ratio \( A_{540}/A_{280} \) of 0.39. A sample of the protein was oxidized by the addition of a slight molar excess of potassium ferricyanide followed by thorough dialysis to remove products. Both oxidized and reduced proteins were concentrated by vacuum ultrafiltration through a collodion membrane. Concentrations were estimated with the extinction coefficients reported by Dus et al. (7).

Variable temperature magnetic susceptibility measurements were made with a superconducting coil vibrating sample magnetometer, designed and constructed by Dr. A. Redfield of the IBM-Watson Laboratories. The experiment was performed...
as follows: 0.75 ml of approximately 3 mm ferredoxin was pipetted into the sample bucket, and 5 μl of 1 mm glucose oxidase and 20 μl of 1 mm glucose were added with stirring. This quantity of glucose oxidase and glucose is more than adequate to remove dissolved oxygen and consume any additional oxygen which might diffuse into the sample container prior to freezing of the sample. This is confirmed by the absence of any paramagnetism resulting from dissolved oxygen in the buffer controls. For temperatures above 4.2° K, the sample bucket was placed in the sample holder assembly and the sample frozen in the freezing mixture of the appropriate temperature. The sample holder assembly was then evacuated thoroughly and inserted into the magnetometer. Although the sample is immersed in liquid helium, it is insulated from the helium by the vacuum jacket and hence remains at the temperature of the freezing mixture. For measurements at 4.2° K and lower, gaseous helium was admitted to the sample as a heat exchange medium. Temperatures below 4.2° K were obtained by pumping on the liquid helium and the temperature determined by measuring the vapor pressure of the helium atmosphere. The susceptibility is determined from the slope of the plot of induced current against applied field with a proportionality factor obtained by measuring the 4.2° K susceptibility of a known quantity of cerium magnesium nitrate, a well defined standard (8). The samples were examined in applied fields up to +30 k gauss in attempts to detect anomalous saturation behavior or the presence of exchange coupled states. At the end of the experiments on the protein the sample was removed, thawed, and reduced by the addition of 1 mg of sodium dithionite plus 1 mg of Tris base. The protein was then refrozen and the measurements repeated.

RESULTS AND DISCUSSION

Fig. 1 illustrates the susceptibility of oxidized and reduced spinach ferredoxin as a function of inverse temperature. Data for a sample of buffer treated similarly to the oxidized protein are also included.

Oxidized spinach ferredoxin exhibits a susceptibility which is independent of temperature and thus has no first order paramagnetism, i.e. no unpaired electrons. The contribution from

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### Table I

<table>
<thead>
<tr>
<th>Iron-Sulfur Proteins</th>
<th>μ\text{eff}^{\text{ox}}</th>
<th>μ\text{eff}^{\text{red}}</th>
<th>Change in μ\text{eff}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinach ferredoxin</td>
<td>0.23 ± 0.05</td>
<td>3.21 ± 0.05</td>
<td>2.98</td>
</tr>
<tr>
<td>Sample I</td>
<td>0.00 ± 0.03</td>
<td>2.84 ± 0.03</td>
<td>2.84</td>
</tr>
<tr>
<td>Sample II</td>
<td>0.65 ± 0.06</td>
<td>3.43 ± 0.06</td>
<td>2.78</td>
</tr>
<tr>
<td>Parsley ferredoxin</td>
<td>0.28 ± 0.03</td>
<td>3.84 ± 0.03</td>
<td>3.56</td>
</tr>
<tr>
<td>Sample I</td>
<td>3.39</td>
<td>0.23</td>
<td>3.16</td>
</tr>
</tbody>
</table>

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second order (temperature-independent) paramagnetism is presumably small because of the close agreement with the susceptibility of the buffer blank.

Reduced spinach ferredoxin possesses a susceptibility which varies linearly with inverse temperature over the range studied (1.4-201° K) and thus obeys the simple Curie law. The slope of the susceptibility agrees well with that expected for a spin ½ system with the known g values (1) of this protein.

Table I summarizes the data for two preparations each of spinach and parsley ferredoxins. All of our samples are either only feebly paramagnetic or diamagnetic in the oxidized form. The small paramagnetism observed in some of the samples is less than 15% of that anticipated for a single unpaired electron, and presumably arises from some trace paramagnetic contamination. This contention is borne out by the observation that one sample (Fig. 1) is unambiguously diamagnetic. It should be borne in mind that the maximum observed residual paramagnetism could be produced if only 1% of the ferredoxin molecules were denatured and the iron in the strongly magnetic high spin ferric (S = ½) state. This possibility is clearly difficult to avoid.

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Fig. 1. The temperature dependence of the volume susceptibility of oxidized and reduced spinach ferredoxin.

Fig. 2. The temperature dependence of the susceptibility of an exchange-coupled pair of high spin ferric ions.

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\[ \mu_{\text{eff}} = (3k/N\mu_B^2)[dX_m/d(1/T)] \] where \( N = \) Avagadro's number, \( X_m = \) molar susceptibility, \( \mu_B = \) Bohr magneton, and \( k = \) Boltzmann's constant. For \( S = 0 \) the theoretical value of \( \mu_{\text{eff}} = 0 \). For \( S = \frac{1}{2} \) and the g values for spinach ferredoxin (1), the theoretical value of \( \mu_{\text{eff}} = 2.84 \). For \( S = \frac{1}{2} \) and the g values for high potential protein (12), the theoretical value of \( \mu_{\text{eff}} = 3.14 \).
It is also important to note that the residual paramagnetism shows an accurate Curie law dependence and thus is not the result of thermal population of excited states of the ferredoxin iron itself.

All four samples give a susceptibility near that of $S = \frac{1}{2}$ on reduction. Deviations from the predicted value are not greater than would be anticipated from the presence of the impurity already discussed, uncertainty in extinction coefficient, possible trace denaturation on addition of dithionite, and pipetting errors. We conclude, then, that these proteins are diamagnetic in the oxidized form and have a net spin of $\frac{1}{2}$ when reduced.

Table I also contains the data for our measured susceptibility of high potential protein. In contrast to the ferredoxins, it is the reduced species of this protein which is diamagnetic, or nearly so. The oxidized protein is paramagnetic and exhibits a value for $\mu_B^2$ in good agreement with that calculated from the measured $g$ values, assuming a single unpaired electron per molecule of protein.

A purely ionic interpretation of the susceptibility data for the two-iron proteins, parsley and spinach ferredoxin, would point to the presence of two low spin ($S = 0$) $\text{Fe}^{II}$ in the oxidized protein, with the reduction of one to a formal $\text{Fe}^{I}$ ($S = \frac{1}{2}$) in the reduced form. However, a model for these proteins (3) has also attributed the diamagnetism of the oxidized form to antiferromagnetic exchange coupling between 2 high spin $\text{Fe}^{III}$ ions. The lack of paramagnetism observed for the oxidized form to 201° K enables us to put a lower limit on the strength of such an exchange coupling. Fig. 2 shows theoretical curves for the susceptibility of the oxidized form as a function of exchange coupling between the iron, expressed as the splitting on the first excited ($S = 1$) state of 2 coupled $S = \frac{1}{2}$ ions. It is evident that the splitting must be greater than thermal energies of 60° K. This would imply a strong, but not unprecedented, coupling of the two species. With strong exchange coupling invoked in the oxidized form, the $S = \frac{1}{2}$ fit to the susceptibility data for the reduced protein can be explained by, for instance, an exchange coupled pair of $S = \frac{1}{2}$, $\text{Fe}^{III}$ and $S = 2$, $\text{Fe}^{II}$ ions.

The lack of paramagnetism does not, of course, permit us to put an upper limit on the strength of the exchange interaction, and it should be noted that the limit of extremely strong exchange coupling is equivalent to considering the oxidation-reduction active site of the protein as a complex with molecular orbitals encompassing both iron centers. The $\text{SFe}$ electron paramagnetic resonance hyperfine structure observed in the reduced form of some other members of this class of non-heme iron proteins suggests that the single unpaired electron does interact equally with both iron atoms (9).

Thus, in the molecular orbital model, the bonding molecular orbitals of the oxidized protein are completely filled, thereby accounting for the diamagnetism of the protein. Reduction places a single electron in the (energetically) lowest antibonding orbital, giving rise to the $S = \frac{1}{2}$ paramagnetic susceptibility and the coupling of both iron atoms to the electronic spin. It should be noted, however, that with spinach ferredoxin it has not been possible to resolve the electron-iron nucleus hyperfine interaction (10), and thus it is not yet possible to determine whether the electron interacts with one or both iron centers in this protein.

Coupling of the various iron atoms also appears to occur in the four-iron high potential protein from Chromatium. This protein exhibits electron paramagnetic resonance in the oxidized form only, with an integrated intensity equivalent to 1 unpaired electron per molecule of protein (11) and Mössbauer spectra suggest that all 4 iron atoms are affected equally by the paramagnetism (12). Our preliminary susceptibility measurements of this protein confirm that it is diamagnetic in the reduced form and show that the oxidized protein contains 1 unpaired electron which, in the light of the Mössbauer result, must be delocalized over all 4 iron atoms.

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REFERENCES
