An Analysis of the Electron Paramagnetic Resonance Spectrum of Pseudomonas oleovorans Rubredoxin

A METHOD FOR DETERMINATION OF THE LIGANDS OF FERRIC IRON IN COMPLETELY RHOMBIC SITES*

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SUMMARY

From thermodynamic measurements, it is possible to identify the ligand atoms bound to Fe$^{3+}$ in nearly rhombic environments. $D$, the second rank axial coefficient in the spin Hamiltonian, is more than 4 times larger for primarily sulfur-ligated than for primarily oxygen-ligated high spin ferric iron.

For the model in this study, rubredoxin, isolated from Pseudomonas oleovorans, containing 1 g atom of Fe$^{3+}$ per mole of protein was used. The electron paramagnetic resonance (EPR) spectrum is that of mononuclear Fe$^{3+}$ in a nearly completely rhombic environment ($E/D = 0.28$). The EPR of rubredoxin containing 2 g atoms of Fe$^{3+}$ per mole of protein is essentially the same; the second atom of Fe$^{3+}$ enters a magnetically equivalent site. At low temperatures, near 1.4°K, the resonance observed in an X-band spectrometer at $g_{eff} = 9.4$ is that of a ground state transition, while the one observed at higher temperatures at $g_{eff} = 4.31$ is that of an excited state transition. The other resonances to higher ($g_{eff} = 4.0$) and lower ($g_{eff} = 4.7$) field of the $g_{eff} = 4.31$ resonance are also excited state transitions but these arise from the two other principal directions. Fitting the amplitude of any feature of the EPR spectrum taken over the temperature range 1.4 to 40°K to a Boltzmann distribution yields the zero field splitting from which $D$ (1.76 cm$^{-1}$ in this case) is determined. Similar variable temperature studies performed on ferric pyrrolidone dithiocarbamate, where Fe$^{3+}$ is completely ligated to sulfur as is rubredoxin, yields a value of 1.68 cm$^{-1}$ for $D$.

High spin ferric iron specifically bound to proteins commonly appears in sites of two different symmetries. In heme iron proteins, the constraints of the ligand system are such that the symmetry of the iron is tetragonal or near tetragonal (1). When iron is specifically bound in a chelate type of structure in non-heme iron proteins such as transferrin (2), ferrichrome (3), or rubredoxin (4-7), the symmetry of the metal atom is found to be nearly completely rhombic (8). In the tetragonal case, the electron paramagnetic resonance spectrum of high spin ferric heme iron is typified by a prominent absorption derivative near $g = 6$ (9), while in the completely rhombic case, the EPR of nonheme iron has a prominent absorption derivative near $g = 4.3$ when examined at X-band.

This communication describes the EPR properties of sulfur-ligated ferric iron in a rhombic site in both a protein and in a model compound. The protein under study, rubredoxin from Pseudomonas oleovorans, when isolated, is found to have a single iron atom per molecule of protein (1 Fe rubredoxin) (10). A second atom of ferric iron can be incorporated into this same molecule (2 Fe rubredoxin) and, as we shall show, enters a magnetically equivalent site. As we shall also show, it is possible from a study of the EPR of rhombic iron taken at various temperatures to describe the ligands to which the iron atom is bound.

MATERIALS AND METHODS

EPR spectra were taken on a superheterodyne X-band spectrometer described previously (11) operating near 9100 Mc per sec and over a temperature range 1.3 to 40°K. For temperatures less than 4.2°K, the EPR cavity was immersed in liquid helium and the temperature was determined from the measured pressure above the coolant. For temperatures above 5.5°K, cooled gaseous helium was blown over the cavity and the temperature was measured with a germanium resistance thermometer which was in intimate contact with the cavity and which was calibrated using liquid helium, liquid H$_2$, and liquid N$_2$ all at atmospheric pressure.

Rubredoxin, containing both a single gram atom of Fe$^{3+}$ (1 Fe$^{3+}$

The abbreviation used is: EPR, electron paramagnetic resonance.
Fig. 1. Energy levels in the three principal directions for *P. oleovorans* rubredoxin using $E = 0.495$ cm$^{-1}$ and $D = 1.76$ cm$^{-1}$ as determined from this study. Arrows indicate energy separations which are of correct magnitude to cause absorption of energy in an X-band EPR spectrometer. The effective $g$ values indicated were those measured for 1 Fe rubredoxin.

Fig. 2. EPR spectra taken at various temperatures for *P. oleovorans* rubredoxin containing a single iron atom per molecule of protein. The spectrometer gains for the different traces have been adjusted arbitrarily. The effective $g$ values for some of the spectral features are indicated on the spectra and the temperature of the measurement is shown for each spectrum.

Fig. 2. EPR spectra taken at various temperatures for *P. oleovorans* rubredoxin containing a single iron atom per molecule of protein. The spectrometer gains for the different traces have been adjusted arbitrarily. The effective $g$ values for some of the spectral features are indicated on the spectra and the temperature of the measurement is shown for each spectrum.

rubredoxin) and 2 gram atoms of Fe$^{3+}$ (2 Fe rubredoxin) per mole of protein, was prepared according to the method of Lode and Coon (10). Ferric tris(pyrrolidone dithiocarbamate) was a gift of Dr. H. H. Wickman and was studied by EPR in a frozen N,N-dimethylformamide solution. Samples (0.7 ml) used for EPR studies contained about 1300 nmoles of Fe$^{3+}$ for 1 Fe rubredoxin, 630 nmoles of Fe$^{3+}$ for 2 Fe rubredoxin, and 1000 nmoles Fe$^{3+}$ for ferric pyrrolidone dithiocarbamate.

RESULTS AND DISCUSSION

For high spin ferric heme iron proteins, the four ligands contributed by the porphyrin of the heme are constrained to lie nearly in a plane, and the EPR spectrum is thus required to show at most only small departures from axial symmetry (11). Usually the second rank interactions in the spin Hamiltonian dominate the fourth rank interactions (the cubic field) and the latter can be neglected. In these cases the magnetic energy levels for iron in the absence of a magnetic field comprise three Kramers doublets, lying at 0, 2$D$, and 6$D$ in energy, where $D$ is the second rank axial coefficient in the spin Hamiltonian (1). The EPR spectrum observed at X-band for high spin heme iron extends from approximately $g = 6$ to $g = 2$ and arises only from the lowest Kramers doublet (11).

For mononuclear high spin ferric nonheme iron proteins, the magnetic levels in the absence of an external magnetic field also comprise three Kramers doublets (3). In the case of completely rhombic symmetry, $E_r$, the second rank rhombic coefficient in the spin Hamiltonian, is equal to $D/3$. In this case the Kramers doublet states are equally separated in energy by an amount $(4\sqrt{7}/3)D$ (Fig. 1). The EPR of iron at X-band in this type of site (Fig. 2) consists of three parts, one from each of the Kramers doublets. If the magnetic field is not too large compared to the energy separation between the Kramers doublets, each of these three parts may be described by a set of three effective $g$ values. The lowest Kramers doublet gives rise to an absorption starting at approximately $g_{eff} = 9$ and extending to much higher magnetic fields ($g_{eff}$ equal to approximately 0.6). The second Kramers
doublet theoretically gives rise to a sharp absorption at $g = 30/7$, and in practice the absorption derivative spectrum observed in these cases consists of a complex pattern centered near $g_{\text{eff}} = 4.3$. The third absorption arising from the highest Kramers doublet is similar to that arising from the lowest and is not usually observed independently of it.

As can be seen in Fig. 2, the EPR of 1 Fe rubredoxin consists of narrow absorptions near $g_{\text{eff}} = 9.4$ and 4.3 and broader absorptions near $g_{\text{eff}} = 4.7$ and 4.0. The low field absorption derivative near $g_{\text{eff}} = 9.4$ represents a magnetic transition arising from the lowest Kramers doublet, but only in one principal direction. To higher field, one can also observe two absorptions, near $g_{\text{eff}} = 1.2$ and 0.9, from the other principal directions and these are observed in both the 1 Fe and 2 Fe rubredoxin samples (Table I). The two high field transitions cannot be resolved in EPR spectra taken at 1.4° K even at the lowest practical operating power of the spectrometer (~10⁻⁸ watts), since the spin lattice relaxation in these directions is very long. At higher temperatures, 5-7° K, these transitions can be observed conveniently at 10⁻⁸ watts. The corresponding transitions from the highest Kramers doublet were looked for at much higher temperatures but were not observed in the protein spectra because of the greatly reduced sensitivity of the EPR spectrometer under these conditions.

The absorption at $g_{\text{eff}} = 4.31$ represents a magnetic transition arising from the middle Kramers doublet but again only in one principal direction. The two EPR absorptions ($g_{\text{eff}} = 4.7$ and 4.0) from the other principal directions to higher and to lower fields of the $g_{\text{eff}} = 4.31$ resonance have greater amplitude and are narrower for the 1 Fe than for the 2 Fe rubredoxins.

At any temperature, the amplitude of the narrow resonance near $g_{\text{eff}} = 4.3$ or the resonance near $g_{\text{eff}} = 9.4$ is directly proportional to the content of iron in both the 1 Fe and the 2 Fe rubredoxin samples under study. This shows that both sites in the 2 Fe rubredoxin are contributing equally to the EPR spectrum.

The EPR absorption arising from each of the Kramers doublets is proportional to the populations of spins in each doublet state and these populations are governed by a Boltzmann distribution. Thus at very low temperatures, where the spin population is greatest in the lowest lying Kramers doublet (Fig. 1), the feature of the EPR absorption having an effective $g$ value near 9 will be the most prominent (Fig. 2). At higher temperatures, where the second Kramers doublet state has a sizable population, the EPR absorption derivative near $g_{\text{eff}} = 4.3$ becomes more prominent and the $g_{\text{eff}} = 9.4$ absorption derivative begins to diminish in intensity (Fig. 2). This temperature dependence is in addition to the usual inverse dependence on temperature exhibited by all EPR absorptions. Thus it is not surprising that the EPR spectrum for rhombic iron in rubredoxin shows both the low field ($g_{\text{eff}} = 9.4$) and the high field ($g_{\text{eff}} = 4.31$) resonances, with the former having greater prominence at lower temperatures.

![Graph](http://www.jbc.org/)

**Table I**

| Kramers doublet | Observed $g$ values | Computed $g$ values* |
|----------------|------------------|--------------------|
|                | 1 Fe  | 2 Fe  |                |
| Lowest         | 9.42  | 9.43  | 9.52            |
|                | 1.22  | 1.16  | 1.23            |
|                | 0.90  | 0.88  | 0.74            |
| Middle         | 4.77  | 4.73  | 4.58            |
|                | 4.31  | 4.30  | 4.20            |
|                | 4.02  | 4.03  | 3.97            |
| Highest        | *     | *     | 9.77            |
|                |       |       | 0.65            |
|                |       |       | 0.41            |

* Computed using $D = 1.76$ cm⁻¹ and $E = 0.495$ cm⁻¹.

* Not observed.

**Fig. 3.** Spin populations of the lowest (upper curves) and middle (lower curves) Kramers doublets for ferrichrome A and for ferric tris(pyrrrolidone dithiocarbamate) (Fe³⁺ TPDG). The value of $D$ (0.50 cm⁻¹) used to generate these curves for ferrichrome A was determined experimentally by Wickman, Klein, and Shirley (3) by studying the temperature dependence of the EPR and also theoretically by detailed analysis of Dowling and Gibson (14), while the value of $D$ (1.08 cm⁻¹) used to separate the curves for tris(pyrrrolidone dithiocarbamate) was determined by the method described in this paper. The data points taken for the $g_{\text{eff}} = 4.3$ resonance for tris(pyrrrolidone dithiocarbamate) are indicated by O.
The upper curves of the figure define the spin populations of the lowest Kramers doublet, the one giving rise to the $g_{\text{eff}} = 9.4$ absorption. The lower curves of the figure define the spin populations of the middle Kramers doublet, the one giving rise to the $g_{\text{eff}} = 4.31$ absorption. At any temperature, the population of spins in the lowest Kramers doublet is greater for the all sulfur ligated ferric system than for the oxygen ligated ferric system.

In order to determine $D$ for 1 Fe and for 2 Fe rubredoxin, as well as for ferric pyrrolidone dithiocarbamate, we examined the temperature dependence of the EPR spectrum taken over the temperature range 1.4 to 40° K. An analysis was performed by taking the product of the amplitude of the $g_{\text{eff}} = 4.3$ resonance and the absolute temperature as a function of the absolute temperature and fitting the data to a Boltzmann distribution over the three Kramers doublets. A least squares fit was made and the splitting between Kramers doublets was determined. Fig. 4 shows such an analysis for 1 Fe and for 2 Fe rubredoxin. The energy splitting between the lowest and middle Kramers doublets was determined as 7.66° K and 7.56° K for the 1 Fe and for the 2 Fe rubredoxin, respectively, assuming completely rhombic symmetry $(E/D = 1/3)$. $D$ was determined as 1.51 cm$^{-1}$ and 1.50 cm$^{-1}$, respectively, and would give rise to the observed energy splitting. Computed curves are given which express the populations of the middle Kramers doublet for both proteins assuming completely rhombic symmetry and these values of $D$. Approximately the same values of $D$ were determined using the amplitudes of the derivative extrema of both the narrow and the broad features to higher and lower fields of the EPR spectrum in the region of $g = 4.3$ and thus these broad and narrow absorptions arise from the same chemical species.

In such a case where there are deviations from completely rhombic symmetry for ferric iron $(E/D \neq 1/3)$, the energy separations between the three Kramers doublets are no longer equal and the change in effective $g$ values reflects this deviation. From a knowledge of effective $g$ values observed in the EPR for the transitions arising from lowest and middle Kramers doublets and the energy separation between these doublets, one can determine deviations from completely rhombic symmetry by solving the second rank spin Hamiltonian for a $d^5$ system (e.g. high spin ferric) exactly (8, 14). In this analysis, $E$ and $D$ were varied until a combination of these terms was found which gave best agreement with the measured effective $g$ values and the thermodynamic determination of the energy splittings between Kramers doublets described above (Table I). This solution changes the previously determined approximate value of $D$ from 1.51 to 1.75 cm$^{-1}$. The agreement between observed and computed $g$ values could be improved by inclusion of the cubic field terms, but, as there are in principle nine of these, the available data is insufficient for such a detailed analysis. Here $E/D$ is equal to 0.28 which is 84% from complete rhombicity. This departure from complete rhombicity (100%) (15) probably reflects the constraint imposed by the protein super-structure on the ferric iron site. If these would not be present, and the ligands had free rotations, it seems likely that $E/D$ would be closer to 0.33 as the stereochemistry of the iron ligand bonds alone would define the symmetry.

These findings can be used to define some aspects of the structure of 1 Fe and 2 Fe rubredoxins. Since the determination of $E$ and $D$ yield essentially the same value in both proteins one must assume that all the iron in the 1 Fe and 2 Fe rubredoxins...
prepared from *P. oleovorans* are in structurally equivalent sites where Fe\(^{3+}\) is ligated to sulfur. Furthermore, the structure maintaining the relative positions of the sulfur atoms bound to iron is indeed the same in both iron binding sites of the 2 Fe rubredoxin protein. This is well in agreement with the x-ray studies of *C. pasteuriunum* rubredoxin where it was shown (16) that Fe\(^{3+}\) is all sulfur ligated.

Thus it is possible from combinations of both thermodynamic and EPR techniques to identify the ligands bound to Fe\(^{3+}\) having close to completely rhombic symmetry in a nonheme iron protein. Since the magnitude of the splittings between the Kramers doublets depends upon the chemical nature of the ligand atoms, one can use this biophysical technique to assign structure and to distinguish between those cases where non-heme ferric iron is primarily oxygen- or primarily sulfur-ligated.

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