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Rubredoxin, a simple iron-sulfur protein: its spin hamiltonian and hyperfine parameters

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Abstract. — Rubredoxin (Rd) from Clostridium pasteurianum is a protein that contains, in its redox active center, a high-spin iron atom coordinated to four cysteine sulfurs of the polypeptide chain. The roughly tetrahedral FeS₄ complex of Rd is the basic structural unit of the active centers in the more complicated iron-sulfur proteins that contain linked clusters of two and four of these tetrahedra.

Under suitable conditions Rd shows Mössbauer spectra with well resolved magnetic and electric hyperfine splittings both in the oxidized and reduced state. These spectra have been parametrized using the spin Hamiltonian

\[ \mathfrak{H} = D[S^2 - S(S + 1)/3 + \lambda(S_H^2 - S_I^2)] + \beta S \cdot g \cdot \mathbf{H} + S \cdot \mathbf{A} \cdot \mathbf{I} + \mathcal{Q} \cdot \mathbf{Q}. \]

At low temperatures the Mössbauer spectra of oxidized Rd, \( S = 5/2 \), consist of a superposition of three resolved component spectra due to the individual Kramers doublets. Only minor relaxation effects are observed in frozen solution at temperatures up to 20 K. The parameters \( D = 2.7 \text{ K}, \lambda = 0.23, g = 2, A = -22.5 \text{ MHz}, \) \( \eta = 0.2 \). Further corrections are discussed. The same Hamiltonian reproduces the 4.2 K, high-field spectra of reduced Rd, \( S = 2 \), with \( D = 10.9 \text{ K}, \lambda = 0.28, g = (2.1, 2.2, 2), A = -3.25 \text{ mm/s}, \eta = 0.65, \) in the slow relaxation limit.

1. Introduction. — Rubredoxins (Rd) are the simplest representatives of the larger class of iron-sulfur proteins, [1] which are widely distributed in nature and are involved in many biological redox reactions. While the active center of the Rd contains a single atom of iron coordinated to four cysteine sulfurs, other iron-sulfur proteins contain linked clusters of two or four FeS₄ tetrahedra [1]. Specifically, the 2 Fe-2 S* proteins have four cysteinylic and two inorganic bridging sulfur ligands, S*, whereas the 4 Fe-4 S* proteins have four cysteinylic and four inorganic sulfurs [2].

The three-dimensional structure of Rd from Clostridium pasteurianum, the protein under study here, has been determined by x-ray diffraction to 1.5 Å resolution [3]. The protein, a single polypeptide chain of 54 amino acids, binds directly to the iron through the thiolate side groups, \(-\text{CH}_2\text{S}^-\), of four characteristically located cysteines. The resulting geometry is roughly tetrahedral, but the differences in bond lengths and angles reduce the local symmetry at the iron site to \( C_1 \). EXAFS measurements on a different Rd are compatible with differences in bond length of up to 0.16 Å [4].

Recently many structural analogs of the iron-sulfur active centers have been synthesized [5], in particular also for oxidized [6] and reduced [7] Rd. Their struc-
tures and electronic properties can be studied much more easily than those of the iron-sulfur proteins that they model, and much can be learned from a systematic comparison of the two.

Because of its importance as the prototype of all iron-sulfur proteins, Rd has been studied extensively by optical [8], magnetic resonance [9, 10] and Mössbauer spectroscopy [10, 11]. It is known, in particular, that oxidized Rd contains high-spin ferric iron, \( S = \frac{5}{2} \), with a characteristic EPR signal near \( g = 4.3 \) [9], and that the iron is in the high-spin ferrous state, \( S = 2 \), in reduced Rd [10]. We hope to demonstrate below that both charge states of Rd offer very favorable conditions for Mössbauer spectroscopy and that it is possible to characterize them in great detail. At low temperatures the spin relaxation of Rd in frozen solution is unusually slow, and the large magnetic hyperfine interactions that are typical for high-spin iron complexes can therefore be observed with high resolution. This is true for reduced as well as for oxidized Rd provided that the former is measured in a strong polarizing field.

To analyse the spectra measured in an external magnetic field \( \mathbf{H} \), assuming the condition \( \beta g H \gg A \) is satisfied, we diagonalize the spin Hamiltonian

\[
\mathcal{H}_S = D[S_x^2 - S(S + 1)/3] + + \beta S \cdot \mathbf{g} \cdot \mathbf{H}, \quad S = 2 \text{ or } 5/2
\]

(1)

to obtain the 2 \( S + 1 \) eigenstates \( |i\rangle \), eigenvalues \( E_i \) and spin expectation values \( \langle S_i \rangle \). Eq. (1) is not the most general form of the spin Hamiltonian and our data on oxidized Rd indeed suggest that additional terms may be needed. As long as the slow relaxation limit applies, we can calculate the Mössbauer spectra in the usual way from the nuclear Hamiltonian \( \mathcal{H}_N \)

\[
\mathcal{H}_N = \langle S_i \rangle \cdot \mathbf{\tilde{A}} \cdot \mathbf{I} - \beta_n g_n \mathbf{H} \cdot \mathbf{I} +
\]

\[
+ (e Q V_{zz} / 12) [3 I_x^2 - 15/4 + \eta (I_x^2 - I_z^2)].
\]

(2)

An integration over all possible orientations of the molecular frame \( (x, y, z) \) and a sum over all \( i \) with appropriate Boltzmann factors \( \exp(-E_i/kT) \) is required. To minimize the number of unknown parameters it is assumed that the quadrupole interaction has the same principal axes \( (x, y, z) \) as the fine structure interaction \( S \cdot \mathbf{\tilde{B}} \cdot S \), the \( g \)-tensor and the \( A \)-tensor. More specific restrictions on \( g \) and \( \mathbf{\tilde{A}} \) will be discussed in the following sections.

A trial and error approach was used to parametrize the data. Using eqs. 1 and 2 spectra were simulated assuming plausible initial parameters and varying them until the simulations agreed well enough with the data. As far as possible the same set of parameters was used to simulate spectra measured under different conditions, e. g. in parallel or perpendicular field. However, even if the parameters pass this test, they should still be compared with other experimental evidence to ascertain their uniqueness. In the case of oxidized Rd, independent information is available from ESR data, while ENDOR experiments and possibly a far-infrared determination of the fine-structure splitting are feasible. For reduced Rd, on the other hand, the possibilities are limited to magnetic susceptibility and, again, far-infrared measurements of the zero-field splitting.

The Rd used in this study was isolated from Clostridium pasteurianum. Samples were enriched in \(^{57}\text{Fe}\) by chemical reconstitution from the apoenzyme. All measurements were done on frozen solutions. The samples were prepared by Dr. W. Lovenberg, N. I. H., Bethesda, MD.

2. Oxidized Rd. — Figure 1 shows the Mössbauer spectra of a frozen solution of oxidized Rd measured in a transverse magnetic field at 1.5 K (a), 10 K (b) and 20 K (c). The spectrum of the same sample in a parallel field of 25 kG at 4.2 K is given in figure 2. The 1.5 K spectrum is practically identical with the data of Rao et al. [11] obtained with a Rd sample from a different species. Inspection of figure 1 and of similar spectra taken in parallel field and at higher temperatures leads to the following conclusions.

![Mössbauer spectra of a frozen solution of oxidized Rd measured in magnetic fields of 1.3 kG perpendicular to the gamma rays a) at 1.5 K, b) at 10 K, c) at 20 K. The solid lines are simulations calculated using the parameters given in Table 1 in the slow-relaxation limit. The velocity scale is relative to Fe metal at room temperature.](image-url)
The data represent a superposition of three distinct component spectra with temperature-dependent intensities. At 1.5 K a single, well resolved six-line pattern is observed with relative intensities that vary little with field direction. From the overall splitting Rao et al. [11] deduced an internal field of 370 kG, and the asymmetry in the splitting implies a small positive quadrupole interaction. At higher temperatures two new components appear at the expense of the low-temperature six-line pattern. One of them is concentrated between \(-3 \text{ mm/s}\) and \(+4 \text{ mm/s}\), consists of broad bands and depends strongly on the direction of the applied field. The last component is another well resolved six-line pattern of larger overall splitting that reaches maximum intensity above 20 K. Like the 1.5 K spectrum it depends only slightly on the direction of the applied field, but in contrast to the former the relevant component of the quadrupole interaction is negative. All these features are readily explained within the framework of eqs. (1) and (2) which will now be specialized for high-spin ferric iron, \(S = 5/2\), and the particular case of Rd. Our analysis follows earlier work of Wickman [12], Lang [13], Spartalian and Oosterhuis [14] and others.

In the ligand-field approach the weak field ground state of ferric iron is an orbital singlet, \(^6A_{1g}\). In the presence of low-symmetry distortions the spin-orbit interaction lifts the six-fold spin degeneracy to give three Kramers doublets. The spin Hamiltonian, eq. (1), with the g-tensor replaced by the isotropic spin-only value \(g_0 = 2.0023\), is generally adequate to describe the magnetic properties of the spin sextet. The Zeeman splittings are easily calculated from eq. (1), and the eigenvalues for \(H = 0\), in particular, are the zero-field energies of the three Kramers doublets. ESR spectroscopists prefer to use a fictitious spin \(S' = 1/2\) representation for each doublet and express the Zeeman interaction in terms of an effective g-tensor \(g^{\text{eff}}\). To lowest order, \(g^{\text{eff}}\) depends on the rhombicity parameter \(\lambda\) only, but is different for each doublet \(k\).

In the oxidized state all Rds show a characteristic ESR signal near \(g = 4.3\) and a single derivative peak near \(g = 9.4\) [9]. The spin Hamiltonian, eq. (1), naturally explains this result if \(\lambda\) is close to its maximum value of 1/3 [9]. The fine structure term in eq. (1) then reduces to \(2/3 D(S_z^2 - S^-^2)\), i.e. it has maximum rhombicity. The effective g-tensors of the lowest and highest doublet are very anisotropic, \(g^{\text{eff}}_1 = (0.86, 9.68, 0.61)\) and, from symmetry, \(g^{\text{eff}}_3 = (0.86, 0.61, 9.68)\). The middle doublet, on the other hand, is magnetically isotropic with a \(g\)-value of \(g = 4.29\). The experimentally observed \(g = 4.3\) signal thus arises from the middle doublet, while the \(g = 9.4\) signal corresponds to the \(y\)-component of the ground state g-tensor \(g^{\text{eff}}_1\). The experimental \(g\)-values are best reproduced with a rhombicity value \(\lambda = 0.28\) [9].

This model not only explains the main ESR results, it also has important consequences for the Mössbauer spectra. We note that the hyperfine tensor \(\mathbf{A}\) in eq. (2) reduces to the isotropic Fermi contact term \(A_0\) for the orbital singlet ground state of high-spin ferric iron. In the lowest and highest Kramers doublet the spin expectation value \(\langle S \rangle\) of the magnetic hyperfine interaction \(A_0\), \(\langle S \rangle\), \(\mathbf{I}\) tend to lie along the \(y\)- and \(z\)-axes, respectively; as a result, large overall splittings are observed in these two doublets with internal fields along the molecular \(y\)- and \(z\)-axes. Since the quadrupole interaction is small relative to the magnetic interaction, only its component along \(\langle S \rangle\) is relevant, i.e. \(V_{zz}\) for the ground doublet and \(V_{yy}\) for the highest doublet. In the middle Kramers doublet, however, which is magnetically isotropic and has a smaller effective \(g\)-value, the spin expectation value follows the direction of the applied field and is roughly half as large as in the other doublets. Since in a frozen solution the molecular frames assume all orientations relative to the applied field and therefore to \(\langle S \rangle\), all components of the quadrupole tensor are sampled and the resulting Mössbauer spectrum is broadened. All these features are noticed in the spectra of figure 1, and our simple model at least qualitatively explains the data.

A quantitative analysis based on computer simulations reveals some problems with this model, though, and the calculated spectra shown in figures 1 and 2 were actually obtained with a slightly modified set of assumptions: (i) Experimentally the overall splitting is found to be 15% larger in the highest Kramers doublet than in the lowest one, and no reasonable choice of rhombicity \(\lambda\) can reproduce this ratio. Of the two assumptions \(g = g_0\) and \(A = A_0\), which apply to an orbital singlet, we dropped the second one, allowing \(\mathbf{A}\) to be a tensor. (ii) The rhombicity parameter \(\lambda\) fixes the ratio of the zero-field
energies of the three Kramers doublets. The choice dictated by the spectra of figures 1 and 2 places the highest doublet at too low an energy. We therefore independently adjust the energies of the higher doublets to best reproduce the data, figures 1a and 1b, while calculating the component spectra due to each doublet from eqs. (1) and (2). In earlier simulations of Rd [15] an additional fine-structure term [14],

\[(D \mu(6)(S_z^2 + S_x^4 + S_y^4 - 707/16)\]

was used which allowed to properly adjust the energies. The resulting parameter set, however, does not reproduce the high-field spectrum, figure 2, and therefore has to be discarded.

Table I lists the parameters used in the final simulations in figures 1 and 2. They should be considered with caution since they have not been optimized yet to simultaneously fit the ESR spectra.

**Table I**

Spin Hamiltonian parameters for oxidized and reduced Rd from Clostridium pasteurianum. Numbers in parenthesis are uncertainties in units of the least significant digit. Values given without uncertainties should be considered preliminary.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Oxidized Rd</th>
<th>Reduced Rd</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\delta^*$ (mm/s)</td>
<td>0.32 (2)</td>
<td>0.70 (2)</td>
</tr>
<tr>
<td>$\gamma$ (mm/s)</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>$\Delta$ (mm/s)</td>
<td>-0.50 (5)</td>
<td>-3.25 (1)</td>
</tr>
<tr>
<td>$\eta$</td>
<td>0.2 (1)</td>
<td>0.65 (10)</td>
</tr>
<tr>
<td>$D$(K)</td>
<td>2.7 (4)</td>
<td>10.9</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>0.23 (2)</td>
<td>0.28</td>
</tr>
<tr>
<td>$g_z$</td>
<td>2.0</td>
<td>2.11</td>
</tr>
<tr>
<td>$g_y$</td>
<td>2.0</td>
<td>2.19</td>
</tr>
<tr>
<td>$g_x$</td>
<td>2.0</td>
<td>2.00</td>
</tr>
<tr>
<td>$A_x$(MHz)</td>
<td>-22.6 (15)</td>
<td>-27.6</td>
</tr>
<tr>
<td>$A_y$(MHz)</td>
<td>-21.8 (4)</td>
<td>-11.4</td>
</tr>
<tr>
<td>$A_z$(MHz)</td>
<td>-23.2 (4)</td>
<td>-41.3</td>
</tr>
<tr>
<td>Electronic</td>
<td>0.0 (†)</td>
<td>4.1 (5)</td>
</tr>
<tr>
<td>state energies</td>
<td>7.1 (15) (†)</td>
<td>22.7</td>
</tr>
<tr>
<td>in zero field (K)</td>
<td>19.6 (20) (†)</td>
<td>46.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48.6</td>
</tr>
</tbody>
</table>

(*) Isomer shift with respect to Fe metal at room temperature.

(†) The parameter $D$ is only properly used in the high magnetic field simulations (Fig. 2). In determining the Boltzmann factors for figure 1, the state energies were used as parameters. The effective $g$ values for the doublets are $g_z = (1.65, 9.27, 1.02)$, $g_y = (3.95, 3.60, 4.90)$, $g_x = (0.38, 0.30, 9.85)$.

3. Reduced Rd. — Reduced Rd has a quadrupole splitting of $|\Delta| = 3.25$ mm/s at 4.2 K which decreases slightly to 3.21 mm/s at 200 K. The isomer shift, $\delta_{Fe} = 0.70$ mm/s at 4.2 K, is typical of high-spin ferrous iron, $S = 2$, with tetrahedral sulfur coordination [16]. Rao et al. [11] observed well resolved magnetic hyperfine interactions in low-temperature, high-field Mössbauer experiments which suggested that a parametrization might be possible. This goal has indeed been achieved as shown by the simulations in figure 3, which are superimposed on spectra taken at 4.2 K in parallel fields of 6.6 kG, 15 kG and 24 kG. The weak temperature dependence of the quadrupole splitting suggests an energy separation of $\sim 900$ K between the orbital singlet ground state of the high-spin ferrous iron and the next higher orbital state [11]. Spin-orbit coupling lifts the fivefold spin degeneracy leading to a fine structure splitting that is assumed to be given by the spin Hamiltonian, eq. (1). The spin expectation value $<S_z>$ is zero for each of the five eigenstates if no field is applied; it increases linearly with $H$ for small fields and finally reaches a saturation value. The magnetic hyperfine interaction observed in the spectra of figure 3 reflects this behavior: It increases more between 0 and 6.6 kG than between 6.6 kG and 24 kG.

The spectra measured in strong field, figure 3b and 3c, consist of well resolved lines rather than of broad bands. Their shape depends very little on the direction.
of the applied field as a comparison shows with the data taken by Rao et al. [11] in perpendicular fields. Such spectra typically arise if the nuclear Hamiltonian, eq. (2), is roughly the same for each molecule irrespective of its orientation. Since the magnetic dipole interaction $<S>\cdot A \cdot I$ is comparable in size with the electric quadrupole interaction and the latter is fixed in the molecular frame, it follows that the spin expectation value $<S>$ must have a preferred orientation in the molecular frame, too. In other words, Rd must have an easy axis of magnetization. As was the case for the high-spin ferric iron, this condition obtains for the top and bottom states of the ferrous $S = 2$ multiplet if the fine structure term has maximum rhombicity, i.e., for $\lambda = 1/3$,

$$S \cdot \vec{D} \cdot S = 2(D/3)(S_x^2 - S_y^2).$$

For positive $D$ the $y$-direction is the easy axis of magnetization, and we therefore expect the spin expectation value to preferentially point along the $y$-axis.

The fact that the high-field spectra contain sharp lines makes it plausible also that the spin relaxation rate is either slow or fast. In the slow relaxation limit each of the five eigenstates of eq. (1) contributes its own component spectrum, weighted by the proper Boltzmann factor. At sufficiently low temperature, however, only the lowest state is populated and the saturation behavior of the magnetic hyperfine interaction $<S>\cdot \vec{A} \cdot I$ seen in figure 3 is determined by the spin expectation value $<S>$ of the ground state only. In the fast relaxation limit, on the other hand, the magnetic hyperfine interaction is determined by the thermally averaged spin expectation value $<S>T$. Since we have been unable to reproduce the experimentally observed saturation behavior in the fast-relaxation limit we conclude that reduced Rd is another example of a slowly relaxing high-spin ferrous complex [17].

Starting from the simple model outlined here the parameters $\lambda$, $\vec{A}$, sign (A) and $\eta$ were optimized; the results are listed in Table I. It should be kept in mind that (i) the spectra analyzed essentially depend on the electronic ground state only, (ii) $\vec{D}$ and $\vec{A}$ were assumed to have the same principal axes as the quadrupole interaction, and (iii) the $g$-tensor was calculated from $D$ and $\lambda$ in second order perturbation. Further measurements are therefore needed to assess the uniqueness of our solution.

4. Summary. — Our results show that it is possible to parametrize the Mössbauer spectra of Rd in terms of the spin Hamiltonian, eq. (1). Some deficiencies of the model are noted for the high-spin ferric case, related in particular to the highest Kramers doublet, which was never before observed directly. Further work is needed to correlate our results with magnetic resonance data. For the high-spin ferrous case the analysis is facilitated by the unusually slow spin relaxation found at low temperature. To check the uniqueness of our parameters, however, higher-temperature Mössbauer spectra must be analyzed, and it is likely that the slow relaxation limit no longer applies under these conditions. The fine structure term $S, \vec{D}, S$, eq. (1), is characterized by a relatively large magnitude $D$ and rhombicity $\lambda$ for both oxidized and reduced Rd. The hyperfine parameters $\vec{A}, \delta$ and $\Delta$ resemble those derived for the Fe$^{3+}$ and Fe$^{2+}$ sites respectively, of reduced 2Fe$-$2S$^*$ proteins [18]. These parameters should serve as a basis for comparison with theoretical models, in particular with molecular orbital calculations [19, 20]. To this end, work on synthetic analogs [6, 21] described elsewhere in this volume, is very important [22-23].

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References