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Mössbauer, EPR, and MCD studies of the C9S and C42S variants of Clostridium pasteurianum rubredoxin and MCD studies of the wild-type protein

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Abstract Rubredoxins contain a mononuclear iron tetrahedrally coordinated by four cysteinyl sulfurs. We have studied the wild-type protein from Clostridium pasteurianum and two mutated forms, C9S and C42S, in the oxidized and reduced states, with Mössbauer, integer-spin EPR, and magnetic circular dichroism (MCD) spectroscopies. The Mössbauer spectra of the ferric C42S and C9S mutant forms yielded zero-field splittings, \( D = 1.2 \text{ cm}^{-1} \), that are about 40 % smaller than the \( D \)-value of the wild-type protein. The \( ^{57}\text{Fe} \) hyperfine coupling constants were found to be ca. 8 % larger than those of the wild-type proteins. The present study also revealed that the ferric wild-type protein has \( \delta = 0.24 \pm 0.01 \text{ mm/s at } 4.2 \text{ K} \) rather than \( \delta = 0.32 \text{ mm/s} \) as reported in the literature. The Mössbauer spectra of both dithionite-reduced mutant proteins revealed the presence of two ferrous forms, A and B. These forms have isomer shifts \( \delta = 0.79 \text{ mm/s at } 4.2 \text{ K} \), consistent with tetrahedral Fe\(^{2+}\)(Cys)\(_4\)(O-R) coordination. The zero-field splittings of the two forms differ substantially; we found \( D = -7 \pm 1 \text{ cm}^{-1} \), \( E/D = 0.09 \) for form A and \( D = +6.2 \pm 1.3 \text{ cm}^{-1} \), \( E/D = 0.15 \) for form B. Form A exhibits a well-defined integer-spin EPR signal; from studies at X- and Q-band we obtained \( g_z = 2.08 \pm 0.01 \), which is the first measured \( g \)-value for any ferrous rubredoxin. It is known from X-ray crystallographic studies that ferric C42S rubredoxin is coordinated by a serine oxygen. We achieved 75 % reduction of C42S rubredoxin by irradiating an oxidized sample at 77 K with synchrotron X-rays; the radiolytic reduction produced exclusively form A, suggesting that this form represents a serine-bound Fe\(^{2+}\) site. Studies in different buffers in the pH 6–9 range showed that the A:B ratios, but not the spectral parameters of A and B, are buffer dependent, but no systematic variation of the ratio of the two forms with pH was observed. The presence of glycerol (30–50 % v/v) was found to favor the B form. Previous absorption and circular dichroism studies of reduced wild-type rubredoxin have suggested d-d bands at 7400, 6000, and 3700 cm\(^{-1}\). Our low-temperature MCD measurements place the two high-energy transitions at ca. 5900 and 6300 cm\(^{-1}\); a third d-d transition, if present, must occur with energy lower than 3300 cm\(^{-1}\). The mutant proteins have d-d transitions at slightly lower energy, namely 5730, 6100 cm\(^{-1}\) in form A and 5350, 6380 cm\(^{-1}\) in form B.

Key words Rubredoxin · Mössbauer · Electron paramagnetic resonance · Magnetic circular dichroism · Radiolytic reduction

Introduction

Rubredoxins are small \((M_r \approx 6000)\) proteins which contain a single [Fe(S-Cys)\(_4\)]\(^{2+}\) site [1, 2]. The function of these proteins is not known; however, they are presumed to serve as electron carriers. In Pseudomonas oleovorans it has been shown that rubredoxin, which in this bacterium is a duplicated \((M_r \approx 12,000\) and two \( Fe \) sites) version of the standard rubredoxin, acts as an electron transfer agent in the hydroxylation reaction of alkanes [3–5]. Various functions have been proposed in a number of bacteria, and several recent reports have provided support for the involvement of rubredoxin in pathways committed to the detection or scavenging of oxygen in anaerobes [6–8].

In terms of their physicochemical properties, rubredoxins are among the best characterized metallopro-
teins. The structural database for this system contains more than 10 amino acid sequences [9] and several X-ray structures, of which four have been refined to resolutions better than 1.2 Å [10–16]. Moreover, a variety of techniques have been used to study the electronic properties of Fe$^{3+}$ and Fe$^{2+}$ sites in rubredoxin [17–34]. The wealth of structural and spectroscopic data (reviewed by Gebhard et al. [35, 36]) that exists for this system, together with the relative simplicity of the monomeric active site, makes rubredoxin an ideal candidate for in-depth studies of the relationships between structure and electronic properties.

Recent advances in the molecular engineering of rubredoxins [37–39] have made possible the investigation of changes in ligand chemistry [37–39], redox properties [40–42], and even modifications of active site nuclearity [38]. Xiao et al. [39] recently reported crystallographic evidence for the presence of an Fe-(O-Ser) bond in the oxidized form of C42S rubredoxin from Clostridium pasteurianum (Cp). The redox potential of the mutant was found to be 200 mV lower than that of the wild-type protein [37, 39]. Xiao et al. also reported that the redox potential as well as the length of the Fe$^{2+}$-(O-Ser) bond depend on pH; the latter information was obtained from EXAFS data on the C42S protein [39]. On the basis of these data it has been proposed that the $\theta$ of the serine ligand probably becomes protonated upon reduction. At low pH the Fe$^{2+}$-O(H)-Ser bond may be broken, which could be the cause of the release of the iron from the protein, as observed in the crystallographic studies [39].

Here we report the results of studies by EPR, Mössbauer, and magnetic circular dichroism (MCD) spectroscopies on the C42S and C9S variants of Cp rubredoxin. In addition to addressing issues concerning Fe(Cys)$_2$(O-Ser) coordination, spectroscopic characterization of the two rubredoxin mutants provide information useful in the identification of serine-coordinated sites in systems of higher nuclearity such as the P-clusters of nitrogenase [43, 44]. The Mössbauer and EPR spectra of oxidized C9S and C42S rubredoxins are very similar to those reported for the wild-type protein, and moreover, only one spectral form is observed for both proteins. Interestingly, however, in contrast to the wild-type protein, chemical reduction of each mutant produces two monomeric ferrous forms, A and B, with very distinct electronic properties. We present here the results of comprehensive spectroscopic studies of the two ferrous forms, including an analysis of the integer-spin EPR spectra of form A at X- and Q-band. Furthermore, we report the results of a frozen-state X-ray irradiation of oxidized C42S rubredoxin demonstrating that at least form A retains the O-Ser coordination, as previously established for the ferric site. We also report MCD data of wild-type rubredoxin and the two mutant proteins (2000–20000 cm$^{-1}$), which reveal well-defined d-d transitions.

### Materials and methods

The genes encoding the C42S and C9S variants of Cp rubredoxin, their expression in Escherichia coli, and the preparation of the $^{57}$Fe-enriched proteins have been described [37, 38]. Unless otherwise specified, all samples were prepared with $^{57}$Fe-enriched proteins in 20 mM Tris-HCl, pH 7.4, 0.2 M NaCl and reduced with a two-fold excess of sodium dithionite. A 0.5 mM solution of C9S rubredoxin was used for the Mössbauer studies of form A. Upon completion of the Mössbauer studies, the same sample was anaerobically thawed and transferred into an X-band EPR tube. The Q-band EPR sample of form A was prepared from a 3 mM solution of C9S rubredoxin. For the Mössbauer studies of form B, glycerol was added to 30% v/v to a 2 mM sample of reduced C42S rubredoxin.

For the pH-dependence studies, three samples were made from a pH 7.4 stock solution of 2 mM reduced C42S rubredoxin. For two samples the pH was changed by adding a fixed volume of either 0.5 M MES (pK$_a$ 6.15) to obtain pH 6, or 0.5 M Tris (pK$_a$ 8.3) to obtain pH 8.5, to a final protein concentration of 0.9 mM. The relative proportions of forms A and B were determined prior to and following pH adjustments using Mössbauer spectroscopy.

“Chloride-free” C9S rubredoxin samples for Mössbauer ($^{57}$Fe) and EPR ($^{57}$Fe) were prepared from a stock solution of the oxidized protein in Tris-HCl buffer. Three successive 10-fold dilutions with 20 mM phosphate buffer, pH 7.6, followed by reconcentrations in an Amicon ultrafiltration device fitted with a YM3 membrane, lowered the salt concentration from 0.2 M to 0.2 mM NaCl; the final protein concentration was 3 mM.

Frozen-state radiolytic reduction was performed on a 2 mM C42S rubredoxin sample in 50% v/v glycerol. The sample was irradiated in an EPR tube at the Cornell High Energy Synchrotron Source (CHESS) following the procedure previously reported [45]. After analysis by EPR, the EPR tube was broken under liquid nitrogen and the sample was transferred into a cup for investigation by Mössbauer spectroscopy.

### Spectroscopy

Mössbauer spectra were recorded with two spectrometers operating in the constant acceleration mode. High-field spectra (0.15–8 T) were recorded using a Janis Research Super-Vari- temp Dewar equipped with a superconducting magnet. Isomer shifts are quoted relative to Fe metal at 298 K. Mössbauer spectral simulations were generated using the WMOSS software package (WEB Research, Edina, Minn.). X-band EPR spectra were recorded using a Bruker ESP 300 spectrometer equipped with an Oxford ESR 910 liquid helium cryostat and an Oxford temperature controller. A Bruker ER 4116 DM dual mode cavity was used to generate the microwave fields, $B_0$, parallel to the static field, $B$. The magnetic field was calibrated with an NMR gaussmeter and the microwave frequency was measured with a Hewlett Packard 5352B counter. Q-band EPR spectra were recorded on a Bruker spectrometer equipped with a locally designed low-temperature microwave probe and cryogenic system [46]. MCD spectra were recorded using an Aviv Associates
41DS circular dichroism spectrometer in conjunction with a Cryomagnetics Incorporated cryomagnet. The contributions arising from natural circular dichroism were subtracted from the spectra by recording data with the applied field in the forward direction, subtracting from this the reverse field data, and dividing the resulting spectrum by two. The CD spectrum of Fig. 10B was constructed by adding the forward and reverse field data and then dividing by two.

Results

Mössbauer and EPR studies of oxidized C9S and C42S rubredoxins

The X-band EPR spectrum of the Fe\(^{3+}\) C42S rubredoxin [37] exhibits, at 4.2 K, two resonances at \(g_{\text{eff}} = 9.7\) and 4.3. Analysis of these \(g\)-values using the \(S = 5/2\) spin Hamiltonian

\[
H_e = D[S_x^2 - 35/12] + E[S_x^2 - S_y^2] + \beta B g S
\]

yields the rhombicity parameter \(E/D = 1/3\). These parameters yield a spin ladder of three equally spaced Kramers doublets with energy separation 3.5\(D\). In the limit of slow electronic relaxation, each Kramers doublet contributes a distinct Mössbauer spectrum with intensities weighted according to the population of the three electronic doublets [47]. Hence, it is possible to deconvolute the measured spectra, establish the Boltzmann population of each doublet, and calculate the zero-field splitting (ZFS) parameter \(D\). We have determined in this way that \(D = 1.22 \pm 0.08\) cm\(^{-1}\) for oxidized C42S rubredoxin.

We have recorded Mössbauer spectra of oxidized C42S and C9S rubredoxins at 45 mT over the temperature range 1.5–20 K. Figure 1B and D shows spectra collected for the C42S mutant at 1.5 K and 10 K, respectively, in a 45 mT magnetic field applied parallel to the path of the \(\gamma\)-radiation. The observation of a well-defined six-line spectrum at 1.5 K (Fig. 1B) shows that at this temperature only the ground Kramers doublet is measurably populated. At 4.2 K (Fig. 1C) and 10 K (Fig. 1D), new features emerge, indicating an increase in the thermal population of higher lying doublets. The arrows in Fig. 1D point to two (of six) transitions associated with the uppermost Kramers doublet.

We have further analyzed the Mössbauer spectra using the Hamiltonian

\[
H = H_e + S \hat{A} I + H_Q - g_\text{N} \beta B I
\]

with

\[
H_Q = eq V_{zz} / 12 \{ 3I_z^2 - 15/4 + \eta (I_x^2 - I_y^2) \}
\]

where \(S \hat{A} I\) describes the magnetic hyperfine interactions of the electronic system with the nucleus, \(H_Q\) describes the interaction of the electric field gradient (EFG) tensor \(V\) with the nuclear quadrupole moment \(Q\) and \(\eta = (V_{xx} - V_{yy})/V_{zz}\).

![Fig. 1 Mössbauer spectra of oxidized C9S (A, hash marks), C42S (B–E, hash marks), and wild-type (E, solid line) rubredoxins recorded with a 45 mT field applied parallel to the \(\gamma\)-radiation. Spectra were collected at 1.5 K (B), 4.2 K (A, C), and 10 K (D, E). The arrows in C point to four (of six) transitions associated with the middle Kramers doublet, while the arrows in D indicate two transitions associated with the upper Kramers doublet. The solid lines in A–D are theoretical simulations generated using the parameters listed in Table 1](image)

For \(E/D = 1/3\) the ground and uppermost Kramers doublets are uniaxial along the \(y\)- and \(z\)-axes of the ZFS tensor, respectively. Owing to this property, the components of the \(A\) tensor along these two perpendicular directions are readily determined. At 1.5 K, only the ground Kramers doublet contributes to the observed spectrum (Fig. 1B); from this spectrum we obtain \(A_y\) and \(V_{yy}\). When the temperature is raised to 10 K, the upper Kramers doublet becomes measurably populated and new features arise in the Mössbauer spectrum. Fitting these features yields \(A_z\) and \(V_{zz}\). With \(V_{yy}\) and \(V_{zz}\) known, \(V_{xx}\) follows from the condition that the EFG tensor is traceless. The last remaining parameter, \(A_x\), was determined by fitting the spectrum of the middle Kramers doublet using the following strategy. Unlike the ground and upper doublets, the middle Kramers doublet is isotropic and consequently the intensities of its spectral features vary with the orientation of the applied magnetic field. For this reason, we collected data at several temper-
Table 1 Spin Hamiltonian parameters for oxidized wild-type, C9S, and C42S rubredoxins

<table>
<thead>
<tr>
<th></th>
<th>WT a</th>
<th>C42S</th>
<th>C9S</th>
</tr>
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<tbody>
<tr>
<td>$D$ (cm$^{-1}$)</td>
<td>1.93</td>
<td>1.22(8)</td>
<td>1.22</td>
</tr>
<tr>
<td>$E/D$</td>
<td>0.23</td>
<td>0.33</td>
<td>0.33</td>
</tr>
<tr>
<td>$A_x$ (MHz)</td>
<td>$-22.6$</td>
<td>$-25(1)$</td>
<td>$-25.3$</td>
</tr>
<tr>
<td>$A_y$ (MHz)</td>
<td>$-21.8$</td>
<td>$-22.1(3)$</td>
<td>$-22.4$</td>
</tr>
<tr>
<td>$A_z$ (MHz)</td>
<td>$-23.2$</td>
<td>$-24.5(3)$</td>
<td>$-24.5$</td>
</tr>
<tr>
<td>$A_{iso}$ (MHz)</td>
<td>$-22.5$</td>
<td>$-23.9$</td>
<td>$-24.1$</td>
</tr>
<tr>
<td>$\Delta E_Q$ (mm/s)</td>
<td>$-0.5$</td>
<td>$-0.7(1)$</td>
<td>$-0.7$</td>
</tr>
<tr>
<td>$\eta$</td>
<td>0.2</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>$\delta$ (mm/s)</td>
<td>$0.24(1)b$</td>
<td>$0.26(1)$</td>
<td>$0.26$</td>
</tr>
</tbody>
</table>

a All parameters, except $\delta$, from Schultz and Debrunner [23]

b This work (see text)

The spectrum recorded for the C9S mutant (10 K) can be fit with the same parameters as obtained for the C42S mutant with only a slight adjustment in the $A$-values.

Fig. 2 Zero-field Mössbauer spectra of forms A (A) and B (B) recorded for the C9S and C42S variants, respectively, at 4.2 K. Spectrum A has been obtained by subtracting a 10% contribution of form B, and spectrum B by subtracting a 15% contribution of form A. The solid lines are simulations generated with the parameters of Table 2.

We have studied a variety of dithionite-reduced samples in different buffers and at different pH values. In all instances we observed two distinct ferrous species with substantially different ZFS parameters. One species, form A, has ZFS parameters such that paramagnetic hyperfine structure is observed in the Mössbauer spectra even in very weak applied fields; in accordance with this observation, form A yields well defined X- and Q-band EPR signals in parallel mode. Form B, on the other hand, requires the application of strong magnetic fields for the development of magnetic features and no EPR signal is observed at X-band. None of the sample preparations yielded pure A or B (with the exception of the radiolytically reduced sample, see below); however, certain conditions produced ca. 90% of either form A or form B, and the spectra associated with the two forms are clearly distinguishable. Thus, we were able to deconvolute the spectra of each form and generate the spectra shown in Figs. 2, 3, 5, and 7 by subtracting 10–45% of the contributions arising from the minority species.

During the course of these studies we noticed that chemical reduction with sodium dithionite favors the formation of form A in C9S rubredoxin, and form B in C42S rubredoxin. Interestingly, however, the measured electronic properties of the forms A and B are essentially independent of the mutant protein (C9S or C42S). This is illustrated in Fig. 3 which shows spectra of form A generated for C9S (hash marks) and C42S (solid line) rubredoxins at 4.2 K and 0.5 T. The spectra can be fit using a single parameter set with only a small adjustment of $\Delta E_Q$, suggesting that the electronic structures of form A derived from the two variants are virtually identical. Similar results were obtained with form B in the two mutant proteins.

Before addressing the conditions which lead to the occurrence of the two forms, we will describe the results of variable-field Mössbauer spectra. For clarity,
we will discuss the Mössbauer spectra of forms A and B and the integer-spin EPR studies of form A separately in the next three sections.

Mössbauer studies of form A

In order to aid the reader with the following discussion, we present in Fig. 4 the ZFS diagrams for forms A and B. Form A has an $S=2$ multiplet for which the ground and first excited magnetic sublevels are split by a small energy $\Delta$. The electronic splittings of this multiplet can be deduced from Eq. 1. Mössbauer studies of form A show that applied fields as weak as 45 mT induce sizeable magnetic hyperfine fields at the $^{57}$Fe nucleus. This behavior is characteristic of an electronic system with integer spin for which the splitting between the two lowest sublevels, $\Delta$, is very small [45, 48]. Simulations of spectra recorded at 0.5 T (Fig. 5A) and at lower fields (data not shown) indicate that $\Delta<0.3$ cm$^{-1}$ and that $D<0$.

We have analyzed the high-field spectra of form A (Fig. 5A–D) using Eq. 2. Simulations to the variable field spectra, discussed below, gave no evidence for rotations of the A tensor relative to the principal axis defined by the ZFS tensor. Therefore, we have kept these tensors collinear. The procedure for fitting variable-field data for high-spin ferrous sites has been described previously, e.g. [49]; thus we will comment only briefly on a few salient features.

The Hamiltonian of Eq. 2 predicts that at 0.5 T the spin expectation values for the lowest spin-level along the x- and y-axes of the ZFS tensor are negligibly

\[ \langle S_x \rangle = -0.06, \quad \langle S_y \rangle = -0.03, \quad \langle S_z \rangle = -2. \]

Under these conditions the magnetic hyperfine field, $B_{\text{int}} = - <S>A/\gamma_B N$, is oriented along the molecular $z$-axis and consequently its magnitude is determined by $S_x A_z$. The best fits to the data were achieved for $A_z = -8.3 \pm 0.1$ MHz.

Figure 5D shows the spectrum of form A recorded at 150 K in a parallel field of 8 T. For $\Delta E_Q > 0$ and $\eta < 0.5$ the high-energy feature between 1 mm/s and 3 mm/s is associated with transitions to the $M_J = \pm 3/2$ levels of the nuclear excited state. These levels are predominantly split when a field is applied along the $z$ direction; thus, the splitting in the spectrum can be matched to $A_z$. Similarly, the “triplet” feature positioned between $-2$ mm/s and 0.5 mm/s arises from transitions to the $M_J = \pm 1/2$ manifold of the nuclear excited state which is split by fields along $x$ and $y$; consequently, simulations to the triplet feature yield $A_x$ and $A_y$. We have simulated the spectrum of Fig. 5D using various combinations of $A_x$ and $A_y$. This

\[ A_z = -8.3 \pm 0.1 \text{ MHz}. \]
Table 2 Spin Hamiltonian parameters for the two forms (A and B) observed upon reduction of the C9S and C42S variants of rubredoxin. For form B the z-axis of the EFG tensor was rotated by $\beta_{\text{EFG}}=13^\circ$ with respect to the z-axis of the D tensor, and the A tensor was rotated by $z_A=18^\circ$ around its z-axis. Numbers in parentheses give estimated uncertainties in least significant digits.

<table>
<thead>
<tr>
<th></th>
<th>Form A</th>
<th>Form B</th>
</tr>
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<tbody>
<tr>
<td>$D$ (cm$^{-1}$)</td>
<td>$-7(1)$</td>
<td>$6.2(13)$</td>
</tr>
<tr>
<td>$E/D$</td>
<td>0.09</td>
<td>0.15</td>
</tr>
<tr>
<td>$g_x$</td>
<td>2.0</td>
<td>2.13$^a$</td>
</tr>
<tr>
<td>$g_y$</td>
<td>2.0</td>
<td>2.18$^b$</td>
</tr>
<tr>
<td>$g_z$</td>
<td>2.08(1)</td>
<td>2.0$^b$</td>
</tr>
<tr>
<td>$AE_Q$ (mm/s)</td>
<td>$2.95(2)$</td>
<td>$-3.27(2)$</td>
</tr>
<tr>
<td>$\eta$</td>
<td>0.42</td>
<td>1.0</td>
</tr>
<tr>
<td>$\beta_{\text{EFG}}$ ($^b$)</td>
<td>$-13$</td>
<td>$-13$</td>
</tr>
<tr>
<td>$A_x$ (MHz)</td>
<td>$-34(5)$</td>
<td>$-16(2)$</td>
</tr>
<tr>
<td>$A_y$ (MHz)</td>
<td>$-31(3)$</td>
<td>$-9.9(5)$</td>
</tr>
<tr>
<td>$A_z$ (MHz)</td>
<td>$-8.3(1)$</td>
<td>$-33(5)$</td>
</tr>
<tr>
<td>$z_A$ (deg)</td>
<td>$-18$</td>
<td>$18$</td>
</tr>
<tr>
<td>$\delta$ (mm/s)</td>
<td>$0.79(1)$</td>
<td>$0.79(1)$</td>
</tr>
</tbody>
</table>

$^a$-values were estimates from the relation $[26] g_{x,y} = g_x^2 + \sqrt{3/(D + E)}$ and $g_z = 2.0$. As argued in the Discussion, this simple relation does not hold for form A and might not hold for form B. The exact choice of $g$ affects the determination of $A$, but should be contained within the quoted uncertainties.

$^b$-values were estimates from the relation $[26] g_{x,y} = g_x^2 + \sqrt{3/(D + E)}$ and $g_z = 2.0$. As argued in the Discussion, this simple relation does not hold for form A and might not hold for form B. The exact choice of $g$ affects the determination of $A$, but should be contained within the quoted uncertainties.

procedure yielded $-38.4$ MHz $\leq A_x \leq -28.8$ MHz and $-32.9$ MHz $\leq A_y \leq -27.4$ MHz.

The parameters obtained from these analyses were refined by performing group fits to the 4.2 K variable field spectra (Fig. 5A–C). This procedure also provided lower and upper limits to the magnitude of the ZFS parameter $D$. For $D = -6$ cm$^{-1}$, the low-temperature data is best fit using $A_x = -28.6$ MHz and $A_y = -26.9$ MHz. These values are near the lower limit allowed by the spectrum of Fig. 5D and thus indicate that $D \leq -6$ cm$^{-1}$. Similarly, $D = -8$ cm$^{-1}$ yielded $A_x = -37.9$ MHz and $A_y = -30.6$ MHz, which are near the lower limit of the range allowed for these parameters. Consequently, $-8$ cm$^{-1} \leq D \leq -6$ cm$^{-1}$. The best fits to the data were obtained using $D = -7$ cm$^{-1}$ and the parameters quoted in Table 2. Simulations generated using these parameters are drawn as the solid lines in Fig. 5.

EPR spectroscopy of form A

The ZFS parameters of form A predict that the two lowest levels of the $S=2$ manifold are separated by $\Delta = 0.18$ cm$^{-1}$, suggesting that EPR signals can be observed both at X-band ($h\nu = 0.3$ cm$^{-1}$) and at Q-band ($h\nu = 1.2$ cm$^{-1}$). Indeed, this form displays a sharp-resonance (ca. 2.5 mT width peak to trough) centered at $B = 68$ mT ($g = h\nu/\beta B = 10$) at X-band (Fig. 6A) and at Q-band a signal is detected at $B = 292$ mT ($g = 8$) (Fig. 6B). The observed $g$-values differ from $4g_z = 8$ (dashed vertical line of Fig. 6) due to a non-zero value of $\Delta$. The resonance condition for an EPR transition between the $l \pm 2$ levels of the $S=2$ manifold is given by $h\nu = \sqrt{\Delta^2 + (4\beta g_z B \cos \theta)^2}$ where $g_z$ is the intrinsic $g$-value of the iron and $\theta$ is the angle between $B$ and the $z$-axis of $D$ [50]. The spectrum is sharpest at $\theta = 0$ because $dB/d\cos \theta$ is at a minimum. The resonance condition then simplifies to $h\nu = \sqrt{\Delta^2 + (4\beta g_z B_z)^2}$ where $B_z$ is the field value at the zero-crossing point of the spectrum. Because form A yields signals both at X-band and Q-band, the two parameters $\Delta$ and $g_z$ could unambiguously be determined from the data, yielding $g_z = 2.08$ and $\Delta = 0.17$ cm$^{-1}$. The value of $\Delta$ agrees with $\Delta = 0.18$ cm$^{-1}$ obtained from Mössbauer spectroscopy.

The broad features observed for integer-spin EPR signals in general imply that the ZFS parameters are distributed. Frequently these distributions are well described by a Gaussian function. However, simulation of the X-band spectrum of form A assuming Gaussian or Lorentzian distributions in $\Delta$ failed to match the spectral lineshape. We have therefore attempted to simulate the data with skewed distributions in $\Delta$ (simulation not shown). While such distributions describe the X-band spectrum quite well, they do not match the resonance observed at Q-band. Alternatively, it is possible that a second species contributes a broad resonance around the $g = 9$ region; in that case it would not be surprising if the Q-band

Fig. 6 Parallel mode X-band (A) and Q-band (B) EPR spectra of form A recorded at 2 K and 6 K, respectively. Spectrum A was obtained for the same sample used to record the Mössbauer spectra of Figs. 2A, 3A, and 5. The X- and Q-band spectra are plotted for equal $g_{eff}$-value scales at the two frequencies. The dashed vertical line represents $g_{eff} = 8$. Instrumental conditions: microwaves, 0.08 mW (A) and 2 mW (B), 9.27 GHz (A) and 34 GHz (B); modulation, 0.49 mT, 100 kHz.
Mössbauer studies of form B

Figure 7 shows 4.2 K Mössbauer spectra of form B recorded at 0.5, 4, and 8 T. It can be seen that form B magnetizes slowly, indicating that the first excited state of the $S=2$ multiplet is substantially removed from the ground level. This observation is consistent with a system for which $D>0$ (Fig. 4B).

Initially we attempted to simulate the variable-field 4.2 K Mössbauer spectra assuming that the EFG and $A$ tensors are collinear with the $D$ tensor. This assumption, however, consistently produced unacceptable fits. We therefore allowed the EFG and $A$ tensors to be rotated relative to the $D$ tensor. The Mössbauer parameters (Table 2) were obtained using a strategy similar to the one described in the previous section.

Solid state radiolytic reduction of C42S rubredoxin at 77 K

As shown above, chemical reduction of the two mutant forms of rubredoxin generates ferrous species with distinct electronic structures. It has been demonstrated in the literature that metalloproteins can be reduced in the frozen state at 77 K by mobile electrons generated by ionizing radiation such as synchrotron X-rays; see, for instance, Davydov et al. [51] and references quoted in that paper. More recently this technique has been successfully applied to a variety of proteins [53, 54, 55, 56]. In our laboratory [45, 52] we have applied this technique to produce the all-ferrous states of [2Fe-2S] and [4Fe-4S] clusters with spectral parameters indistinguishable from those obtained by chemical reduction of the samples at room temperature followed by freezing. Although radiolytic reduction at 77 K may leave the metal center in constrained nonequilibrium conformation, previous studies have suggested (see refs. 22–28, 34, and 40–46 quoted by Davydov et al. [51]) that the coordination geometry remains essentially unaltered. Since the X-ray structure of the oxidized C42S rubredoxin has established that Ser42 coordinates to the ferric ion, one should be able to assess by irradiating oxidized C42S rubredoxin at 77 K whether form A or form B of the reduced state have serine coordination.

A 0.5 T Mössbauer spectrum of radiolytically reduced C42S rubredoxin is shown in Fig. 8. Spectral contributions of oxidized centers (25%) have been subtracted; the remaining iron has spectroscopic features characteristic for a ferrous site. Comparison of the spectrum of radiolytically reduced C42S rubredoxin (hash marks) with the spectrum of form A (solid line) shows that the irradiation produces form A, and little, if any, form B. The EPR spectrum of the irradiated sample is identical to that of form A (data not shown), providing further evidence that the form A generated chemically and the radiolytically generated ferrous site are essentially the same. As radiolytic reduction generates exclusively form A, this form must arise from a metal site having a geometry close to that of the oxidized protein.

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2 The small difference between the spectra of form A of dithionite- and radiolytically-reduced C42S rubredoxin can, for instance, be accounted for by a 1.5% change of $A_1$. 
Buffer and pH effects on the relative ratios of forms A and B

During the course of this investigation we have performed Mössbauer and EPR measurements on ca. 20 samples of the C9S and C42S proteins prepared in different buffers between pH 6 and pH 9. Below we present the results of these studies which: (1) exclude the possibility of chloride and buffer molecules ligating to the iron in form B; (2) suggest that the structural difference between forms A and B most likely does not arise from the differential binding of protonated and deprotonated serine ligands; and (3) indicate that the presence of glycerol stabilizes the structure associated with form B.

The solid lines of Fig. 9 represent a 0.5 T spectrum of dithionite-reduced C42S rubredoxin prepared in Tris-HCl buffer, pH 7.4. The hash marks in Fig. 9A and B show the spectral contributions of form B for samples prepared in a 50:50 mixture of Pipes and Bicine (Fig. 9A) and in phosphate (Fig. 9B) buffers. The contributions of form A (30% in Fig. 9A and 10% in Fig. 9B) have been subtracted and the identity of the spectra of form B shows that the electronic structure of this species is independent of the chemical composition of the buffer, which rules out ligation by a buffer molecule.

Samples of C42S rubredoxin in Tris buffer and in 50 : 50 v/v Pipes/Bicine buffers have also been studied at several pH values. In the Tris-buffered samples at pH 6, 7, 4, and 9 we observed 48%, 43%, and 85% form A, respectively. For the Pipes/Bicine-buffered samples the corresponding proportions of form A were 8%, 30%, and 20% at pH 6, 7, 4, and 9, respectively. These results do not provide evidence for a systematic dependence of the A:B ratio on pH and indicate that the ratio is buffer dependent in a way currently not understood. (The temperature dependence of the pKₐ of the buffers, ≈ −0.8 for Tris and ≈ −0.45 for Pipes/Bicine between 25°C and 0°C, is too small to affect these conclusions in a significant way.)

Finally, we would like to comment on the effect of glycerol on the proportion of the two ferrous forms. In samples prepared in the presence of 30–50 % v/v glycerol, 65–90 % of the reduced centers were found to attain form B (data not shown). In order to separate the effect of glycerol from variations of the equilibrium of A and B arising from different protein preparations, we studied a sample of reduced C42S rubredoxin to which glycerol was added. In this experiment, glycerol shifted the proportion of form B from 50% to 85%. We repeated this experiment with a C42S rubredoxin sample to which 50% v/v glycerol was added in the oxidized state, followed by reduction with synchrotron X-rays at 77 K. Thawing and refreezing this sample³ yielded an 80% conversion from form A to form B. Taken together, these results suggest that glycerol stabilizes the structure associated with form B.

MCD studies of ferrous wild-type, C42S, and C9S rubredoxins

Eaton and Lovenberg [2] have reported the room-temperature electronic spectral properties of both ferric and ferrous Cp rubredoxin. The studies of the dithionite-reduced protein revealed bands at energies higher than 25000 cm⁻¹ (<400 nm) and lower than 8000 cm⁻¹ (>1250 nm) but none in the intervening spectral range. Bands detected at 6000 cm⁻¹ and 7400 cm⁻¹ were assigned to two d-d transitions and the authors also suggested the presence of a third transition at 3700 cm⁻¹. Subsequent low-temperature MCD studies [57, 58] have confirmed the presence of analogous (unassigned) transitions in the ultraviolet region and the absence of bands in the visible region of ferrous Desulfovibrio gigas rubredoxin.

The 4.2 K MCD spectrum of wild-type ferrous rubredoxin (Fig. 10A) contains a positive feature, of ca. 2000 cm⁻¹ width, centered at 6000 cm⁻¹ and no detectable signals in the 8000–20000 cm⁻¹ range (not shown). The 4.2 K natural CD spectrum of the same sample exhibits a maximum at 6000 cm⁻¹ and a minimum at approximately 7000 cm⁻¹ (Fig. 10B). The latter feature is 300–400 cm⁻¹ red shifted with respect to the band present in the previously reported room-temperature CD spectrum of wild-type rubredoxin [2]. These observations suggest that the CD spectrum

³ In order to test the possibility that the composition of the reduced C42S samples is randomly affected by freezing, we anaerobically thawed, stirred, and refroze a Mössbauer sample with well-characterized composition; no changes were observed in the composition of the resulting sample
results from positive and negative bands that overlap and partially cancel each other. As the temperature is lowered to 4.2 K, the bands sharpen leading to a change in the extent of cancellation which in turn shifts the position of the resulting negative component. If this interpretation is correct, the negative CD band must represent a transition centered at, or below, ~7000 cm⁻¹. Thus, contrary to the suggestions of earlier authors [2], the present results do not support the presence of a band at 7400 cm⁻¹ in the spectrum of wild-type rubredoxin.

The MCD spectrum of the native protein shows also that there is no electronic transition detectable at 3700 cm⁻¹. The dotted line in Fig. 10A represents a simulated Gaussian band centered at 3200 cm⁻¹. The tail of this band is barely concealed within the noise in the experimental data, and a band of similar or greater intensity contributing between 3300 cm⁻¹ and 3700 cm⁻¹ would thus be detected as an upwards shift in the scatter of data points present between approximately 3500 cm⁻¹ and 4000 cm⁻¹. The room-temperature absorption-difference spectrum (reduced minus oxidized, not shown) displays a strong maximum centered around 3700 cm⁻¹, but its absence from the MCD spectrum (Fig. 10A) strongly suggests that it must be vibrational in origin⁴. On the other hand, if one of the E₈→T₂₈ transitions were to be centered at an energy lower than about 3300 cm⁻¹ we could not detect it by conventional cryogenic MCD. Because the protein samples are prepared in hydroxyl solvents which display strong O-D stretching mode absorptions (Fig. 10C), a cut-off occurs in the spectrum at energies lower than ~3500 cm⁻¹.

As the Fe site in rubredoxin has a distorted tetrahedral coordination, the most reasonable interpretation of the MCD data is that two (see below) of the three nominally E₈→T₂₈ transitions are within the envelope centered at 6000 cm⁻¹. The solid line drawn through the data of Fig. 10A is a simulation using two Gaussian bands centered at 5900 cm⁻¹ and 6300 cm⁻¹.

Figure 11 shows 4.2 K near-IR MCD spectra of the ferrous C9S (A, constructed spectrum, see the caption of Fig. 11) and C42S (B, measured spectrum) rubredoxin. EPR studies performed on aliquots of these samples indicated that ca. 90% of the C42S variant was in form B while the A:B ratio for the C9S variant was 60:40. Compared to the wild-type protein, the d-d bands of the two forms are red shifted. The solid

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⁴ In principle, a purely z-polarized electronic transition would not be detectable by MCD either. However, it is unlikely that a d-d transition in a site with no real symmetry could exhibit such a transition.
line in Fig. 11 is a simulation assuming two transitions with the energies as listed in the caption of Fig. 11. No MCD bands were observed for either mutant in the visible region of the spectrum.

The spectral envelope of Fig. 11B might be better fit by simulations employing three transitions. However, we have chosen not to do so because the presence of three d-d transitions around 6000 cm\(^{-1}\) would yield 10Dq values substantially different from well-characterized model complexes (see below). The relatively small deviations of the data from a two-transition simulation could be due to an indeterminate instrument error or due to vibronic components in the signal.

**Discussion**

We have studied the EPR, Mössbauer, and MCD spectra of \(\text{Cp rubredoxin and its C42S and C9S variants. Such data on the simplest Fe-S sites shed light on the understanding of serine ligation in more complex members of this category of metal sites.}

As shown in Table 1, C9S and C42S rubredoxins have smaller zero-field splittings in the oxidized state and their average A-values, \(A_{\text{av}} = (A_x + A_y + A_z)/3\), are about 8% larger than that reported for the wild-type protein [23]. The magnetic anisotropy, most probably due to anisotropic covalency, is similar in the mutant and wild-type proteins. Our Mössbauer spectra of the oxidized wild-type protein can be fit well with the parameter set reported by Schulz and Debrunner [23], provided the isomer shift is changed from the reported \(\delta = 0.32 \text{ mm/s} \) to \(0.24 \pm 0.01 \text{ mm/s}\). The new value for \(\delta\) is more consistent with \(\delta = 0.25 \text{ mm/s}\) reported for ferric desulfuredoxin, a protein that also has Fe(Cys)\(_4\) coordination [59], and \(\delta = 0.25 \text{ mm/s}\) [27, 29] observed for an Fe\(^{3+}\)-rubredoxin model complex.

Reduction with sodium dithionite produces two distinct Fe\(^{2+}\) forms (A and B) in samples of both C42S and C9S variants. The electronic parameters of the two forms are essentially the same in both mutants; this is not surprising because the two substituted residues are counterparts in the two-fold symmetry around the metal site (however, see below) [17]. The isomer shifts \(\delta = 0.79 \text{ mm/s}\) of both forms are distinctly larger than \(\delta = 0.70 \text{ mm/s}\) observed for the Fe(Cys)\(_4\) coordination of the wild-type protein, as expected for the replacement of one of the sulfur ligands by an oxygen.

In the absence of crystallographic data for reduced (Fe\(^{2+}\)) C42S Rd, the structures associated with forms A and B cannot be discussed in detail. However, the X-ray structure of oxidized (Fe\(^{3+}\)) C42S Rd [39] provides a working model for form A at least, since the latter has been generated by radiolytic reduction at 77 K, a temperature at which significant structural rearrangements are unlikely\(^5\). In contrast, form B is produced by reduction in solution, and therefore a wider range of structural variations would be possible. One may consider loss of serine ligation, as this ligand is expected to become more labile upon reduction of the iron, possible protonation, and its replacement by an aquo (or other solvent molecule) ligand. However, such an occurrence would presumably destabilize the metal site, whereas experiments have shown that C9S and C42S Rd can undergo several redox cycles without detectable decomposition. Other experiments have shown that neither pH nor chemical composition of the buffer have systematic effects on the A:B ratio. The A:B ratio, however, is dependent on the presence of cryoprotectants (glycerol), and on the variant (C9S or C42S) considered. These observations suggest that A and B might have only slightly different structures originating from the oxidized C42S framework. The latter differs from the wild-type site by the replacement of a 2.23 Å Fe-S bond by a 1.94 Å Fe-O bond, while other bond lengths remain largely unmodifed [39]. Addition of an electron to this asymmetric structure may give rise to multiple conformations in solution, of which two may be trapped at cryogenic temperatures. The vicinity of solvent molecules could explain the effect of solvent composition on the ratio of A:B. Also, slight differences between the Cys9 and Cys42 sites, in particular with respect to hydrogen bonding [15], might explain why the A:B ratios of the C9S and C42S variants differ. Clarification of these questions would require crystallographic structures of the reduced states of C42S and C9S Rd.

We do not know the identity of the non-cysteinylligand in form B; however, we have eliminated chloride and buffer molecules as possible candidates. Since the isomer shift of form B is the same as that of form A, an oxygen has likely replaced the mutated cysteine sulfur in form B also. Our studies of the possible pH dependence of A and B interconversion are not conclusive. However, based on the observation of a weak and inconsistent dependence of the ratio of the two species on pH, and the fact that this ratio clearly depends on factors other than pH, it seems unlikely that the structural distinction between forms A and B arises from differential binding of the iron to deprotonated and protonated serine residues, respectively. Conceivably, form A and B could represent protonated iron-bound serine residues forming hydrogen bonds with different bases (this might explain the weak pH dependence). Another possibility, namely that form A is serine coordinated (protonated or deprotonated) and that form B represents a water-coordinated site, is suggested by the EXAFS results of Xiao et al. [39] (who, however, were not aware that the reduced variant rubredoxins contain mixtures of two different species).

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\(^5\) The geometry of the metal site of the C42S variant, as determined using the coordinates deposited in the Protein Databank (entry 1BE7), differs somewhat from that given in [39]. For instance, the Fe-O-Ser distance determined with the actual coordinates (1BE7) is 1.94 Å, whereas it is given as 1.82 Å in [39].
The particular values of the fine structure parameters $D$ and $E$ for form A have allowed us to observe a well-defined integer-spin EPR signal at X- and Q-band. From these studies we obtained $g_z = 2.08 \pm 0.01$ for the $z$-component of the electronic $g$ tensor. If one considers only the $^3D$ term of the high-spin ferrous ion, the ZFS parameters $D$ and $E$, for $D < 0$, are related to $g_z$ by [50]

$$g_z \approx 2.00 + (2k/\lambda) (|D| + |E|)$$

(4)

where $\lambda$ is the spin-orbit coupling constant and $k$ is the isotropic orbital reduction factor [60]. Inserting our results for form A, namely $D = -7 \pm 1$ cm$^{-1}$, $E/D = 0.09$, and $g_z = 2.08 \pm 0.01$, into Eq. 4 and using $\lambda = -100$ cm$^{-1}$ (from [61]) yields a rather low value for $k$, namely $k \approx 0.5$. Because a smaller value for $D$ would yield, in our view, a more reasonable value for $k$, we have considered whether fourth-order terms in the spin Hamiltonian might yield an overestimation of $D$. In the presence of fourth-order terms we can write the expression for the two lowest spin levels (Fig. 4A) as $A = A_2 + A_4$ where $A_4$ is a contribution by fourth-order terms. Equation 1 for $B = 0$ yields the expression

$$\Delta_2 = 2D(1 - \sqrt{1 + 3(E/D)^2}).$$

However, the expression for $A_2$ assumes that fourth-order terms in the spin Hamiltonian (coefficients $a$ and $F$; see [61]) are negligible. In principle, fourth-order terms could contribute substantially to the $A$-value of form A, allowing one to reduce $A_2$ and therefore $D$. However, while the low-field ($B < 0.2$ T) M"ossbauer spectra and the EPR spectra are sensitive to the quartic terms, the high-field ($B > 4$ T) M"ossbauer spectra depend essentially on $D$ and are quite insensitive to $A$. Thus, $D$ of form A remains confined to $-8 \leq D \leq -6$. Moreover, there are other substantial reasons to question the validity of Eq. 4. Low-lying $^3T$ states of the ferrous ion can mix with the $^3E_{g}$ ground states and thus contribute to $D$. Solomon and co-workers have estimated for the Fe(RS)$_3^-$ anion that the low-lying $^3T$ states contribute $-1.5$ cm$^{-1}$ to $D$ [36] and spin-spin interactions are expected to contribute ca. $-0.5$ cm$^{-1}$ to $D$ [62]. Subtracting both estimates from our $D$-value would yield a more reasonable $k \approx 0.75$. In summary, it seems likely that the presence of low-lying $^3T$ states (14000–18000 cm$^{-1}$), demonstrated for tetrahedral sites in Fe(RS)$_2^2$- and Fe$^{2+}(\text{ZnS})$ [63], may provide a sizeable contribution to the $D$-value of C42S rubredoxin. Therefore, the use of Eq. 4 is questionable for form A and, by extension, also for wild-type rubredoxin. This is unfortunate because ZFS parameters are relatively easy to determine while the three $g$-values of ferrous ion are generally not obtained from EPR, Mössbauer, or MCD experiments. If Eq. 4 cannot be used, it is difficult to estimate the orbital contribution to the isotropic part of the ferrous A tensor; this contribution is proportional to $|1/3 \text{Tr}(g)| - 2$.

We have recorded near-IR low-temperature MCD spectra for C42S and C9S Rd as well as for the wild-type protein. The MCD spectra of C9S and C42S Rd, shown in Fig. 11A and B, exhibit a broad feature whose center is shifted by ca. 200 cm$^{-1}$ to lower energy relative to the wild-type protein. The band shapes suggest that this feature contains at least two $E_g \rightarrow T_{2g}$ transitions. The small shifts compared to the wild-type protein suggest only minor changes in the ligand field. Our data for the wild-type protein yield d-d transition energies different from those deduced from room-temperature absorption and CD studies by Eaton and Lovenberg [2]. These authors have concluded that the wild-type protein exhibits a band at 7400 cm$^{-1}$. Clearly, the low-temperature MCD spectra lack a feature at this energy. Eaton and Lovenberg also suggested the presence of a band centered around 3700 cm$^{-1}$. Our data suggest that this band, if present at all, occurs at an energy below 3300 cm$^{-1}$. The question thus arises whether one, two or three $E_g \rightarrow T_{2g}$ transitions contribute to the feature centered at 6000 cm$^{-1}$. If only two transitions are contained in the $=6000$ cm$^{-1}$ band, the third d-d transition would have to be below 3300 cm$^{-1}$.

Solomon and co-workers reported a comprehensive single-crystal MCD study of a complex, Fe(SR)$_4^2-$ (R = 2-PhC$_6$H$_4$), with tetrahedral thiolate coordination [36]. That study yielded 10Dq = 3500 cm$^{-1}$, the same value as reported for Fe$^{2+}$ doped into tetrahedral ZnS (3500 cm$^{-1}$ [63]). Based on this work and the following interpretation, we propose that the 6000 cm$^{-1}$ feature observed for wild-type rubredoxin (Fig. 10A) most likely consists of only two $E_g \rightarrow T_{2g}$ transitions. This feature around 6000 cm$^{-1}$ reflects transitions from the ground state to two sublevels of the $^5T_{2g}$ manifold, with transition energies that depend on the tetrahedral splitting (10Dq) as well as on the axial and rhombic splittings of the $^5E_g$ and $^5T_{2g}$ manifolds. From a very limited set of published quadrupole splittings for ferrous Rd, $\Delta E_O = 3.26$ mm/s at 4.2 K and 3.21 mm/s at 200 K [23], the splitting of the $E_g$ states can be estimated to be 600–800 cm$^{-1}$, implying an unreasonably large 10Dq = 5800 cm$^{-1}$ if we were to associate three $E_g \rightarrow T_{2g}$ transitions with the feature around 6000 cm$^{-1}$. Thus, in order to obtain for ferrous rubredoxin a 10Dq value in the range of that observed for the model compound and Fe$^{2+}(\text{ZnS})$, at least one $E_g \rightarrow T_{2g}$ transition must be well below 3300 cm$^{-1}$. In principle, analysis of the ZFS and A tensor data of ferrous rubredoxin should give information regarding the energy of the low-energy $E_g \rightarrow T_{2g}$ transition. However, the Mössbauer spectra of ferrous Rd [23] have only been studied in applied fields smaller than 2.5 T, and consequently $D$, $A_x$, and $A_z$ have very large uncertainties (we have estimated from the published data that $D$ is uncertain by about $\pm 40\%$).
Conclusions

The changes observed upon individually mutating the Cys42 and Cys9 residues of *C. pasteurianum* rubredoxin to serine can be summarized as follows:

1. In the oxidized state the two variants display δ-values (δ = 0.26 mm/s) which are 0.02 mm/s larger than that of the wild-type protein (δ = 0.24 mm/s). The average A-values, \( A_{av} = (A_x + A_y + A_z)/3 \), are also about 8% larger.

2. Reduction of each of the two variants yields two high-spin ferrous sites (form A and form B) with distinct electronic structures. These two forms are obtained under conditions where the reduction of the variants is reversible, and their electronic structures depend neither on the composition of the solvent nor on the identity of the variant. In contrast, the A:B ratio depends on the variant, on the chemical composition of the solvent, and, though not in a systematic way, on the pH. The A form must retain the Fe(5-Cys)₃(O-Ser) coordination sphere, since it is obtained also by radiolytic reduction of the oxidized C42S protein at 77 K. The active site geometry of form B has remained elusive and its elucidation will require further studies. Although a change of coordination cannot be ruled out, the parameters governing variations of the A:B ratio suggest that slight changes in the environment of the metal site, possibly in hydrogen-bonding and orientation of the serine ligand, might account for the A→B conversion.

3. The isomer shifts \( \delta = 0.79 \) mm/s of both the A and B forms are distinctly larger than \( \delta = 0.70 \) mm/s observed for the Fe(Cys)₄ site of the wild-type protein.

4. MCD studies of the wild-type and the two mutant forms of the protein yielded well defined d-d transitions. In combination with the MCD studies of a model complex published by Solomon and co-workers [36], we conclude that two d-d transitions of the wild-type and variant proteins are clustered around 5000–6500 cm⁻¹, while the remaining transition most likely contributes to energies lower than 3300 cm⁻¹. These conclusions provide an important benchmark in the study of the electronic properties of Fe₄S₉ sites.

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