Mössbauer Studies of *Escherichia coli* Sulfite Reductase Complexes with Carbon Monoxide and Cyanide

EXCHANGE COUPLING AND INTRINSIC PROPERTIES OF THE [4Fe-4S] CLUSTER*

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Mössbauer studies of the hemoprotein subunit (SiR) of *E. coli* sulfite reductase have shown that the siroheme and the [4Fe-4S] cluster are exchange-coupled. Here we report Mössbauer studies of SiR complexed with either CO or CN and of SiR in the presence of the chaotropic agent dimethyl sulfoxide (Me₂SO). The spectra of one-electron-reduced SiR-CN show that all five iron atoms reside in a diamagnetic environment; the ferroheme-CN complex is low spin and the [4Fe-4S] cluster is a 2.07 signal. Titration with ferricyanide affords a CN⁻ complex of oxidized SiR in which the siroheme iron is low spin ferric, with the cluster remaining in the 2+ state. At low temperatures, paramagnetic hyperfine interactions are observed for the iron sites of the cluster, suggesting that it is exchange-coupled to the heme iron. Reduction of one-electron-reduced SiR-CN and SiR-CO yields complexes with \( g = 1.94 \) \( ^{-} \)-type EPR signals showing that the second electron is accommodated by the iron-sulfur cluster. The fully reduced complexes yield well resolved Mössbauer spectra which were analyzed in the spin Hamiltonian formalism. The analysis shows that the clustersite is equivalent in pairs, one pair having properties reminiscent of ferric sites whereas the other pair has features more typical of ferrous sites.

The Mössbauer spectra of oxidized SiR kept in 60% (v/v) Me₂SO are virtually identical with those observed for SiR in standard buffer, implying that the coupling is maintained in the presence of the chaotrope. Fully reduced SiR displays an EPR signal with \( g \) values of \( g = 2.53, 2.29, \) and \( 2.07 \). This \( g \) signal vanishes with a change in the spin state of the heme iron from \( S = 1 \) (or 2) to \( S = 0 \).

The NADPH-sulfite reductase of *Escherichia coli* is a large (\( M_r \approx 885,000 \)) oligomeric protein (\( \Theta_{\text{dimer}} \)) which catalyzes the 6-electron reductions of sulfite to sulfide and of nitrite to ammonia. The substrate-binding site resides on the \( \beta \)-subunit (termed SiR) which contains one siroheme, an isobacteriochlorin, and one [4Fe-4S] cluster per mol. SiR can be isolated as a monomer by treatment of the holoenzyme with 4 M urea followed by DEAE chromatography. When provided with a suitable artificial electron donor such as reduced methyliodogen, SiR is catalytically competent.

Using the deazafavin-EDTA photooxidation system originally described by Massey and Hemmerich (1), Janick and Siegel (2) have shown that SiR is readily reduced by up to 2 electrons. The first added electron isaccommodated by the siroheme and results in the loss of the \( S = \frac{3}{2} \) EPR signal characteristic of oxidized SiR. Addition of a second electron yields an EPR-active species with \( g \) values at \( g = 2.53, 2.29, \) and \( 2.07 \) (-0.65 spin/SiR) and two minority \( S = \frac{3}{2} \) species (together -0.15 spin/SiR). The addition of 60% (v/v) Me₂SO to fully reduced SiR produces drastic changes in the optical spectrum with a concomitant appearance of a strong \( g = 1.94 \) EPR signal.

We have demonstrated (3, 4) that the siroheme and the iron-sulfur cluster are exchange-coupled in the three oxidation states of SiR and in a turnover complex of SiR with nitrite (a ferroheme-NO intermediate). These studies have shown that Mössbauer spectroscopy is uniquely suited to study this novel system. Here we have extended those studies to complexes of SiR with carbon monoxide and cyanide. Optical and EPR data (5, 6) have shown that these ligands bind stoichiometrically and that they coordinate to the heme prosthetic group. Complexes with CN⁻ can be prepared in states containing either a ferri- or a ferroheme, whereas CO binds only in those states where the heme iron is ferrous.

Studies of the reduced inhibitor complexes are of particular interest for the following reason. In a low spin ferrous state, the siroheme iron is not suitable for the development of interatomic exchange. Therefore, the spectroscopic features of the two prosthetic groups are affected only in minor ways by the coupling between them. Thus, these states provide us with an opportunity to study the intrinsic spectroscopic properties of the [4Fe-4S] cluster. Such information is indispensable for an understanding of the exchange-coupling phenomena in SiR.

**MATERIALS AND METHODS**

_E. coli_ K12 cells were grown as previously described (7), with 0.5 mg/liter \(^{55}\)Fe (95% enrichment, New England Nuclear). Sulfite reductase hemoflavoprotein complex was purified as described by Siegel et al. (7) and SiR was isolated using the method of Siegel and Davis (8). The enzyme was buffered in 50 mm potassium phosphate at pH 7.7. Enzyme concentration was determined using an absorbance of 1.8 x 10^4 M⁻¹ cm⁻¹ (9). All of the studies reported in this work were performed with \(^{55}\)Fe-enriched enzyme.

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1 The abbreviation used is: Me₂SO, dimethyl sulfoxide.
Mössbauer samples of SiR ligated to CO or CN\textsuperscript{−} were prepared by partially photoreducing anaerobic solutions of 250–300 μM SiR, 50 μM deazaflavin, 10 mM EDTA, and 1 mM CN\textsuperscript{−} or 0.6 mM CO in EPR tubes to allow rapid binding of the ligand to the siroheme. The partially reduced SiR-CN samples (at least 1-electron reduced) were either directly transferred to a Mössbauer cup in a Thunberg tube under Ar or fully oxidized by addition of ferricyanide (added in small aliquots while the optical spectrum was monitored) before transfer. The samples were frozen in liquid nitrogen. The oxidation state of the complex was determined optically by comparison with spectra obtained during a ferricyanide titration of fully reduced SiR-CN (see Fig. 2 of Ref. 5). Since photoreduction of SiR-CN past the 1-electron state was very slow, the fully reduced SiR-CN sample was prepared by addition of dithionite (2 mM final concentration) to the EPR tube, followed by anaerobic transfer to a single Mössbauer cup in a Thunberg tube under Ar and then frozen in liquid nitrogen. For SiR-CO, 150 μl of the partially reduced SiR-CO was transferred to each of two Mössbauer cups in a Thunberg tube under Ar and an anaerobic EPR tube. The samples were further illuminated until complete reduction was achieved as determined using the optical spectrum of the sample in the EPR tube. The samples were then frozen in liquid nitrogen for EPR and Mössbauer analysis. The two Mössbauer cups were placed back to back in the spectrometer for analysis. (A single 300-μl sample would have been optically too dense to permit complete photoreduction.)

Optical spectroscopy was performed using an Aminco DW-2 dual beam spectrophotometer. EPR spectra were recorded as described previously (2, 5, 10) using a Varian E9 spectrometer equipped with an Air Products He gas transfer cryostat. Spin concentrations were determined by quantitation with a cupric EDTA standard. The Mössbauer spectrometers used were of the constant acceleration type, as described in the accompanying paper (4). The isomer shifts, δ, are quoted relative to the centroid of the spectrum of iron metal recorded at room temperature.

RESULTS

**Sulfite Reductase Complexed with Carbon Monoxide**—Murphy et al. (11) have shown that CO binds tightly to the siroheme iron of reduced SiR. Since CO is a strong field ligand, the siroheme iron of SiR\textsuperscript{2−} -CO is expected to be low spin ferrous (S<sub>i</sub> = 0). In the fully reduced state, SiR\textsuperscript{2−} -CO,\textsuperscript{2} the complex exhibits an EPR signal of the g = 1.94 type with g values at 2.029, 1.926, and 1.910 (5). This signal, which is characteristic of the 1+ oxidation state of the [4Fe-4S] cluster, accounts for 1 spin/siroheme.

Fig. 1 shows a Mössbauer spectrum of SiR\textsuperscript{2−} -CO recorded at 4.2 K in zero field. The spectrum exhibits a sharp quadrupole doublet superimposed on a broad component. The latter belongs to the paramagnetic [4Fe-4S]\textsuperscript{1+} cluster. In general, one records spectra of paramagnetic species in applied magnetic fields H > 10 milliTeslas. In zero field, such spectra tend to be broader and more poorly resolved. We have used this to our advantage; because the [4Fe-4S]\textsuperscript{1+} cluster spectrum is broad, the spectrum of the siroheme appears as a conspicuous quadrupole doublet in Fig. 1. By matching two Lorentzian lines to the doublet, we found that it accounts for ~20% of the total absorption, i.e. the doublet has the proper intensity to be assigned to the siroheme-CO complex. The observed isomer shift δ = 0.23 ± 0.02 mm/s is typical of ferrous heme-CO complexes. The value for the quadrupole splitting, ΔE<sub>Q</sub> = 0.80 ± 0.03 mm/s, is somewhat larger than those observed for other heme-CO complexes (see Table 9 of Ref. 12, and Ref. 13). The parameters obtained here strongly implicate a low spin ferrous siroheme.

Since low spin ferrous hemes have no thermally accessible excited electronic states, their spectra have temperature-independent quadrupole splittings. Within the resolution of our data, the siroheme-CO complex fits this pattern. Since the spectrum of the siroheme-CO is well defined, its contribution can be reliably subtracted from the raw data to yield the spectra of the [4Fe-4S]\textsuperscript{1+} cluster.

Fig. 2 shows spectra of the [4Fe-4S]\textsuperscript{1+} cluster recorded at 4.2 K in external magnetic fields of 60 milliTeslas (A and B)
and 6.0 Teslas (C). The spectra (discussed below) exhibit magnetic hyperfine interactions, as expected for an EPR-active species with slow electronic spin relaxation. At higher temperatures, above 50 K, the relaxation rate becomes fast compared with the nuclear precession frequencies. Consequently, the magnetic hyperfine interactions are averaged out and only quadrupole doublets are observed. A representative spectrum, taken at 110 K, is shown in Fig. 3A. The solid line indicates the 4.2 K spectrum of the siroheme-CO (determined from Fig. 1), shifted by \(-0.03 \text{ mm/s}\) to account for the second order Doppler shift and scaled to represent 20\% of the total Fe. The spectrum in Fig. 3B was obtained by subtracting this heme contribution from the raw data. The remaining spectrum belongs to the [4Fe-4S] cluster; it consists of two quadrupole doublets, labeled I and II. The solid line is the result of fitting four Lorentzians to the data. The observation of two doublets of equal area suggests that the four sites of the iron-sulfur cluster are equivalent in pairs. At 110 K, site I has \(\Delta E_q(I) = 0.70 \pm 0.03 \text{ mm/s}\) and \(\delta(I) = 0.47 \pm 0.02 \text{ mm/s}\); whereas site II has \(\Delta E_q(II) = 1.80 \pm 0.30 \text{ mm/s}\) and \(\delta(II) = 0.59 \pm 0.02 \text{ mm/s}\). The average isomer shift referred to 4.2 K, \(\delta_{\text{av}} = 0.56 \text{ mm/s}\), is characteristic of \(\text{Fe}^{2+}\) in the 1 oxidation state; in the oxidized, 2+ state, [4Fe-4S] clusters exhibit shifts of about 0.45 mm/s at 4.2 K (see below). The quadrupole splittings of sites I and II, obtained at various temperatures, are listed in Table I.

A detailed analysis of the 4.2 K spectra displayed in Fig. 2 shows that the four sites of the iron-sulfur cluster are still equivalent in pairs even when the magnetic hyperfine interactions are taken into account. The solid lines in Fig. 2 are the result of fitting each of the two distinct sites to the spin Hamiltonian

\[
H = \beta S \cdot g \cdot H + \sum_i \mathbf{I}_i - g_i \mathbf{I}_i \cdot \mathbf{B} \cdot I
\]

In Equation 1, \(S = \frac{1}{2}\) is the cluster spin and \(g\) the electronic g tensor, known from EPR. \(A\) is the magnetic hyperfine tensor and \(V_{zz}, V_{xy}\), and \(V_{xz}\) are the principal axis components of the electric field gradient tensor; \(\eta = (V_{zz} - V_{xy})/V_{zz}\) is the asymmetry parameter; \(\Delta E_q = (e Q V_{zz} \eta^2) (1 + \eta^2/3)^{1/2}\) is the quadrupole splitting. The parameters obtained from a series of computer simulations are listed in Table II.

In the following, we discuss briefly the salient features of the spectra; for this discussion it is assumed that \(A\) and \(g\) are isotropic. In the presence of an applied field \(H\), each \(^{57}\text{Fe}\) nucleus senses an effective field \(H_{\text{eff}}\) which is the sum of an internal field \(H_{\text{int}}\) and the applied field; \(H_{\text{int}} = H_{\text{eff}} + H\). (For a discussion, see Ref. 13.) The internal magnetic field is given by \(H_{\text{int}} = -A \cdot \langle S \rangle /g_i\beta_i\), where \(\langle S \rangle\) is an appropriately taken expectation value of the electronic spin. For \(\text{SiR}^{2-}\cdot\text{CO}\), \(\langle S \rangle = \pm \frac{1}{2}\) for an arbitrary direction \(i\); the signs refer to the \(M = +\frac{1}{2}\) (spin up) and \(M = -\frac{1}{2}\) (spin down) substates of the electronic ground state. An applied field of 6 Teslas splits the electronic ground state by an energy \(E/k_B \approx 5\) K. Thus, at 4.2 K, essentially only the \(M = -\frac{1}{2}\) state is populated (87\%). Associated with this state is an internal field \(H_{\text{int}} = +A/g_i\beta_i\).

A comparison of the spectra of Fig. 2, B and C, shows that they contain a component whose magnetic splitting increases with increasing applied field, i.e. \(H_{\text{int}} > H_{\text{int}}\) and therefore \(A > 0\). Virtually all absorption at Doppler velocities around \(-2 + 3\text{ mm/s}\) in Fig. 2C belongs to this component. Spectral simulations (see dashed curve) readily reveal that two equivalent iron sites are associated with this component. Moreover, the simulations suggest, but do not rigorously prove, that the component with \(A > 0\) gives rise to doublet II in the spectrum of Fig. 3B. This suggestion has strong support from Mossbauer studies of \(D.\) gigas ferredoxin II: this protein contains a 3Fe cluster which can be converted into a [4Fe-4S] cluster in the presence of \(^{57}\text{Fe}\), sulfide, and dithiothreitol (15). The \(^{57}\text{Fe}\) is incorporated into a subsite which gives rise to doublet II at 90 K and to a magnetically split component at 4.2 K with features very similar to those of component II discussed here (see Figs. 7 and 9 of Ref. 15).

The well defined outer features of the 6.0-Tesla spectrum of component II determine its parameters to a large extent. Detailed computer simulations revealed that the magnetic hyperfine tensor must be anisotropic, and that \(\Delta E_q > 0\). After a reasonable set of parameters was found, the theoretical

![Fig. 3. 110 K Mossbauer spectra of \(\text{SiR}^{2-}\cdot\text{CO}\). The spectrum in A was taken in zero field. The solid line indicates the 4.2 K spectrum of the siroheme-CO moiety. Subtraction of this spectrum from the data yields the 110 K spectrum of the [4Fe-4S]\(^{1+}\) cluster, as shown in B. The solid line in B is a fit to the data assuming two doublets of equal intensity, labeled I and II.](http://www.jbc.org/)

**Table I**

| Quadrupole splittings of the [4Fe-4S]\(^{1+}\) cluster of \(\text{SiR}^{2-}\cdot\text{CO}\) The labels I and II correspond to those in Figs. 2 and 3. The values in parentheses give uncertainties in units of the least significant digit. |
|---|
| Site I | Site II |
| \(T\) | \(\Delta E_q\) | \(\delta\) | \(\Delta E_q\) | \(\delta\) |
| K | (mm/s) | (mm/s) | (mm/s) | (mm/s) |
| 195 | 0.68 (3) | 0.44 (2) | 1.68 (3) | 0.54 (2) |
| 110 | 0.70 (3) | 0.47 (2) | 1.80 (3) | 0.59 (2) |
| 70 | 0.75 (3) | 0.47 (3) | 1.88 (6) | 0.61 (3) |

**Table II**

| Spin Hamiltonian parameters used to simulate the 4.2 K spectra of the [4Fe-4S]\(^{1+}\) cluster of \(\text{SiR}^{2-}\cdot\text{CO}\) The parameters are compared with those obtained by Middleton et al. (14) for the \(B.\) steaurothermophilus ferredoxin. The values in parentheses are estimates of the uncertainties of the least significant digit. |
|---|
| Site I | Site II |
| \(\delta\) (mm/s) | 0.49 (3) | 0.62 (3) | 0.50 (2) | 0.58 (2) |
| \(\Delta E_q\) (mm/s) | 0.72 (7) | 0.32 (4) | 26.5 (6) |
| \(\eta\) | 0.70 (6) | 1.89 (6) | 32.8 (5) |
| \(A_1\) (MHz) | -31.6 (14) | -19.3 (14) | -31.7 (14) |
| \(A_2\) (MHz) | -31.7 (14) | -22.0 (14) | -32.8 (5) |
| \(A_3\) (MHz) | -31.7 (14) | -11.0 (28) | -27.8 (14) | +8.6 (8) |
Sulfite Reductase Complexes with Carbon Monoxide and Cyanide

spectra representing component II were subtracted from the experimental data of Fig. 2. The remaining spectra were fitted with one set of parameters, i.e. component I contains, like component II, the contributions of two equivalent iron sites. The magnetic splitting of component I decreases in an applied field, i.e. A < 0. Moreover, the analysis showed that the A tensor of component I is quite isotropic and that its components are larger in magnitude than those of spectral component II. We will argue below that the irons of site I are somewhat ferric in character whereas those of site II have features of Fe(II).

For the data analysis, we have assumed that all tensors in Equation 1 have the same principal axis system. Since the g values are virtually isotropic, it is not possible to correlate the components of g and A. Furthermore, since the A tensor of site I is isotropic, its components cannot be correlated with the electric field gradient tensor. For site II, however, the components of the field gradient tensor can be correlated with those of the anisotropic A tensor; the field gradient components are adjustable by the value for \( \eta \). The spectra displayed in Fig. 2 show that the overall agreement between experiment and theory is quite good; thus, we felt no need to rotate the A tensor of site II relative to the field gradient tensor.

Once reasonable values for the parameters were obtained, we performed an extensive series of simulations aimed at optimizing the parameter set with respect to the whole set of experimental data. These calculations also yielded reasonable estimates for the uncertainties; the estimates are based on visual inspections and are quoted in Table II.

Sulfite Reductase Complexed with Cyanide—Using optical and EPR spectroscopy, Janick and Siegel (5) have recently studied the complexes of SiR with cyanide in all three oxidation states. These studies suggest the following picture: SiR–CN contains a low spin \( S = \frac{1}{2} \) ferric siroheme and a \([4Fe-4S]^{+}\) cluster. The first electron reduces the heme at a midpoint potential of \( E_m = -155 \text{ mV} \) (6) rendering it low spin ferrous \( (S = 0) \). The second electron which enters the complex at a substantially lower potential, \( E_m = -490 \text{ mV} \) (6), is accommodated by the iron-sulfur-cluster reducing it to the 1+ state, as witnessed by the appearance of a \( g = 1.94 \) signal.

Fig. 4A shows a Mossbauer spectrum of SiR–CN taken at 4.2 K in an applied field of 60 millitelsas. The sample contained 10% of SiR–CN, the contribution of which we have removed from the spectra of Fig. 4. The spectrum consists of one sharp quadrupole doublet, showing that all four Fe sites of the cluster as well as the siroheme iron have essentially the same values for \( \Delta E_Q \) and \( \delta \). The observed isomer shift, \( \delta = 0.45 \pm 0.02 \text{ mm/s} \), unambiguously shows the cluster to be in the 2+ oxidation state. Furthermore, the value of \( \delta \) and \( \Delta E_Q = 1.00 \pm 0.03 \text{ mm/s} \) are the same as those observed for the cluster of oxidized and 1-electron-reduced SiR (3, 4), of SiR–NO (4) and SiR–CN (see below); in all these states, the cluster is in the 24+ state. The quadrupole splitting observed here is independent of temperature for 1.5 K \( \leq T \leq 200 \text{ K} \). The spectrum shown in Fig. 4B was taken at 4.2 K in a field of 6.0 Teslas. The solid line is a theoretical spectrum computed with the assumption that all five iron sites are diamagnetic. The triplet structure of the 6.0-Tesla spectrum results from a powder average of a diamagnetic species with \( \Delta E_Q = 1.0 \text{ mm/s} \) whose nuclear experience an effective field of 6.0 Teslas, i.e. \( H_m = 0 \). The positions of the three major bands are practically fixed by the magnitude of \( \Delta E_Q \) and the value of \( H_m \). The excellent agreement between the theoretical curve and the data shows that SiR–CN is a diamagnetic compound. In a mirror way, the spectrum depends on the signs of \( \Delta E_Q \) and the values of the asymmetry parameters \( \eta \). Values for \( \eta < 0.7 \) produce a shoulder on the low energy side of the central band if \( \Delta E_Q > 0 \) whereas the opposite is observed when \( \Delta E_Q < 0 \). Clearly, the majority of the iron sites of SiR–CN must have \( \Delta E_Q > 0 \). It is reasonable to assume that these sites belong to the \([4Fe-4S]^{+} \) cluster, since positive \( \Delta E_Q \) values are also suggested from the analyses of the SiRo (3) and SiR–NO (4) spectra. For the fit displayed in Fig. 4B, we used \( \Delta E_Q > 0 \) for all sites of the iron-sulfur cluster, together with \( \eta = 0 \) for two cluster subsites and \( \eta = 0.5 \) for the other two sites. For the siroheme iron, we used \( \Delta E_Q < 0 \) and \( \eta = 0.5 \). Since we are dealing with a multiparameter problem, the \( \eta \) values are not uniquely determined.

The fully reduced cyanide complex, SiR–CN, exhibits a \( g = 1.94 \)-type signal with \( g \) values at 2.03, 1.93, and 1.91, values almost identical with those observed for the corresponding CO complex. We have prepared a sample by adding dithionite, as described above. Examination of the Mossbauer spectra revealed that the sample contained about 15% of SiR–CN. After removing this contribution from the raw data, we obtained spectra very similar to those shown in Figs. 2 and 3B. As an example, 4.2 K spectra of the cluster as observed in SiR2–CO. A spectrum taken under the same conditions as that shown in Fig. 1 revealed that the siroheme-CN moiety contributes a 1.94 signal larger in magnitude than those of spectral component I1. For the data analysis, we have assumed that all tensors in Equation 1 have the same principal axis system. Since the g values are virtually isotropic, it is not possible to correlate the components of g and A. Furthermore, since the A tensor of site I is isotropic, its components cannot be correlated with the electric field gradient tensor. For site II, however, the components of the field gradient tensor can be correlated with those of the anisotropic A tensor; the field gradient components are adjustable by the value for \( \eta \). The spectra displayed in Fig. 2 show that the overall agreement between experiment and theory is quite good; thus, we felt no need to rotate the A tensor of site II relative to the field gradient tensor.

Once reasonable values for the parameters were obtained, we performed an extensive series of simulations aimed at optimizing the parameter set with respect to the whole set of experimental data. These calculations also yielded reasonable estimates for the uncertainties; the estimates are based on visual inspections and are quoted in Table II.
Sulfite Reductase Complexes with Carbon Monoxide and Cyanide

Fig. 5. 4.2 K Mössbauer spectra of fully reduced SiR complexes taken in 60-milliTesla parallel fields. A, spectra of the [4Fe-4S]2+ cluster of SiR2–CN (hashmarks) and SiR2–CO (full circles), the spectral contributions of the heme were removed from the data (see text). B, spectrum of SiR2– in 60% Me2SO. The doublet of the siroheme iron is marked by the bracket. For comparison, the spectrum of the [4Fe-4S]2+ cluster of SiR2– CO is shown (circles), plotted as 80% of the total iron.

Fig. 6. Mössbauer spectra of SiR2–CN recorded at 195 K in zero field (A) and at 4.2 K in a parallel field of 60 milliTeslas (B). The solid line in A is the result of a least squares fit, assuming two quadrupole doublets constrained in the intensity ratio of 4:1. The solid line in B is the result of a computer simulation using Equation 1 for the spectrum of the [4Fe-4S]2+ cluster; the spectrum has been scaled to 80% of the total absorption and its base-line has been displaced downward to facilitate comparison with the data. The dashed curve is a spectral simulation for the low spin ferric heme-CN complex in the framework of the ligand field model described in the text.

CN is shown in Fig. 6A. It consists of a superposition of two doublets. The majority component, accounting for 80% of the total absorption, belongs to the [4Fe-4S] cluster; its Mössbauer parameters are the same as in all states where the cluster is at the 2+ oxidation level. The low energy line of the doublet belonging to the siroheme-CN moiety is clearly discernible at ~0.5 mm/s Doppler velocity; the high energy line superimposes almost perfectly with the high energy line of the iron-sulfur cluster. From a least squares fit to the spectrum (solid line in Fig. 6A), we obtained \( \Delta E_0 = 1.35 \pm 0.08 \text{ mm/s} \) and \( \delta = 0.19 \pm 0.05 \text{ mm/s} \) at 195 K. At 90 K, the quadrupole splitting is slightly larger, \( \Delta E_0 = 1.44 \pm 0.08 \text{ mm/s} \). The values for \( \Delta E_0 \) and \( \delta \) agree very well with those typically observed for protoporphyrin-cyanide complexes (see Table 4 of Ref. 17) and those reported for the cytochrome a-CN complexes of the Thermus thermophilus (18) and the beef heart cytochrome oxidases (see Footnote 7 in Ref. 18).

A low temperature spectrum of SiR2–CN at 4.2 K in a 60-milliTesla parallel field is shown in Fig. 6B. We will focus first on the siroheme spectrum. A comparison of the spectra in Fig. 6, A and B, shows that the quadrupole doublet of the siroheme has vanished and that a broad magnetic component has appeared. The lack of resolution precludes a quantitative evaluation of this component. We can, however, compare the shape and the overall splitting of the spectrum with the features predictable from the EPR data and with information reported for other heme-CN complexes. We have therefore calculated theoretical spectra in the framework of the ligand field model proposed by Griffith (19) and further developed by Blumberg and Peisach (20) and Oosterhuis and Lang (21). In this model, the g values determine the ratios \( \Delta/\lambda \) and \( V/\lambda \), where \( \Delta \) and \( V \) are tetragonal and rhombic distortion parameters, respectively, and where \( \lambda \) is the spin-orbit coupling constant. The observed g values yield \( \Delta/\lambda = 1.79 \), \( V/\lambda = 2.95 \), and \( k = 0.71 \) for the orbital reduction factor. From these parameters, and using \( P = -(4.2 \text{ mm/s})/2 = -1.8 \text{ mm/s} \) to scale the A tensor (for definitions, see Refs. 17 and 21), we have computed the theoretical spectrum (dashed curve) shown in Fig. 6B. Clearly, the experimentally observed spectrum for the siroheme iron is not in disagreement with the theory.

Comparison of the spectra in Fig. 6, A and B, shows that the absorption lines of the [4Fe-4S]2+ cluster are noticeably broadened at 4.2 K. For a structure such as the [4Fe-4S]2+ cluster, one would observe temperature-dependent widths for the absorption bands if the \( \Delta E_0 \) values of the subsites had different temperature dependencies. In general, \( \Delta E_0 \) values change when excited electronic states become thermally accessible. This mechanism would be more likely to cause broadening of the spectra at higher temperatures. Here, however, the broadening appears at very low temperatures. Here, however, the broadening appears at very low temperatures. Moreover, the [4Fe-4S]2+ clusters observed for SiR2– and SiR2–NO-CN have quadrupole doublets with temperature-independent \( \Delta E_0 \) values and lines which remain sharp at 4.2 K. Significantly, for the SiR2–CN we observe broadening of the cluster spectrum at low temperatures concomitant with the appearance of magnetic hyperfine interactions in the siroheme spectrum. Thus, we are led to the conclusion that the broadening observed in Fig. 6B is caused by the presence of magnetic hyperfine interactions. Since the broadening persists in zero field, the cluster belongs to a system of half-integer spin. For SiR2– and for SiR2–NO, we demonstrated (3, 4) that the magnetic hyperfine interactions of the cluster subsites \( (\nu = 1–4) \) can be described by \( A_i S_i \bar{I} \), where \( S_i \) is the spin of the siroheme and where \( A_1 \approx A_2 \approx -A_3 \approx -A_4 \). It seems reasonable to assume that the hyperfine interactions observed for the cluster of SiR2–CN can be described in a similar manner. The theoretical curve (solid line) extending from ~0.8 to +1.8 mm/s in Fig. 6B was generated using Equation 1 and the g values quoted above, together with \( A_1 = A_2 = -A_3 = -A_4 = 2.3 \text{ MHz} \). This description yields a reasonable representation of the
broadening. We have also studied the sample at 4.2 K in a 6.0-Tesla field. The [4Fe-4S] cluster spectrum was well described by two components with effective magnetic fields of 5.2 and 6.9 Teslas, corresponding to internal fields in agreement with the above quoted A values. We will argue below that the magnetic hyperfine broadening of the cluster absorption lines reflects exchange interactions between the siroheme and the iron-sulfur cluster.

The data presented here show that the siroheme is low spin ferrous in both SiR2−.CN and SiR2+.CN. Interestingly, the Mössbauer parameters of the siroheme iron are different for these states. For SiR2+.CN, we obtained ΔE = 0.72 ± 0.05 mm/s and δ = 0.39 ± 0.03 mm/s at 4.2 K; these parameters could be extracted quite easily from the zero field spectrum. Precise parameters of the siroheme doublet of SiR2−.CN are more difficult to determine since the doublet is completely masked by the contribution of the [4Fe-4S] cluster. Since SiR2−.CN and SiR2+.CN both have the iron-sulfur cluster in the 2+ state, we have produced a difference spectrum by subtracting the 140 K spectra of the two samples. This procedure should cancel the contribution of the iron-sulfur cluster. From an analysis of the resultant difference spectrum (22), we obtained for the siroheme-CN moiety of SiR2+.CN the values ΔE = 0.9 ± 0.1 mm/s and δ = 0.42 ± 0.05 mm/s.

Taking the second order Doppler shift into account, this isomer shift was extrapolated to δ = 0.45 mm/s at 4.2 K. Since the spectrum of SiR2+.CN exhibits a temperature-independent quadrupole splitting, we can assume that the siroheme-CN moiety has ΔE = 0.9 mm/s at 4.2 K as well. Thus, the siroheme-CN complex has different values for ΔE in SiR2+.CN and SiR2−.CN; the isomer shifts, however, are the same within the uncertainties.

Oxidized and Fully Reduced SiR in 60% Dimethyl Sulfoxide—Janick and Siegel (5) have reported EPR and optical studies of SiR in the presence of Me2SO in various concentrations. In the presence of 60% Me2SO, the optical spectrum of SiR3+ is the same as that observed in standard buffer; the EPR spectra show a slight shift in γ values from γ = 6.63, 5.24, and 1.98 to γ = 6.54, 5.35, and 1.93, i.e., the rhombicity parameter of the zero-field splitting term shifts from E/D = 0.829 to 0.825. The Mössbauer spectra (22) of the 60% Me2SO sample are virtually identical with those reported (3) for the native enzyme. The small differences observed can be attributed entirely to the differences in E/D. Since the two chromophores are exchange-coupled in SiR2+, the similarity of the spectra observed in standard buffer and in 60% Me2SO solution implies that the siroheme and the iron-sulfur cluster remain exchange-coupled in the presence of 60% Me2SO.

The addition of 60% Me2SO to SiR2− changes the EPR and the optical spectra dramatically (5). The g = 2.29 and the S = 1/2 species observed in SiR2− (2) disappear and the only observable EPR-active species exhibits a g = 1.94-type signal with γ = 2.03a, 1.928, and 1.905 (see Table I of Ref. 5). A Mössbauer spectrum of a 60% Me2SO sample recorded at 4.2 K in a 60-milliTesla field is shown in Fig. 5B (hashmarks). The spectrum is a superposition of a magnetically split component which is identical with the spectrum (full circles) of the [4Fe-4S]3+ cluster of SiR2−.CO, and of a quadrupole doublet (bracket) with Mössbauer parameters ΔE = 1.45 ± 0.05 mm/s and δ = 0.44 ± 0.02 mm/s with ΔE independent of temperature for T < 200 K. These parameters suggest strongly that the siroheme iron is low spin ferrous. A conclusion which agrees with the interpretation of the optical spectra (5). In the absence of Mössbauer data of suitable siroheme model complexes, an interpretation of the values obtained for ΔE and δ in terms of ligand structure is not yet possible. While it is conceivable that Me2SO occupies the sixth coordination position of the heme iron, the strength of this ligand is probably insufficient to affect a transition to a low spin configuration (23, 24). More likely, the chaotropic Me2SO has perturbed the protein conformation sufficiently to allow an endogenous ligand to gain access to the heme iron.

DISCUSSION

We have demonstrated previously (3) and in the accompanying paper (4) that the siroheme and the iron-sulfur cluster are exchange-coupled in SiR3+ (in standard buffer and in 60% Me2SO), in SiR2−, in SiR2+, and in the turnover complex, SiR2−.NO (in standard buffer as well as in 2 M urea). The data obtained here suggest that the coupling is also maintained in SiR2−.CN. The arguments supporting this claim are as follows. The presence of paramagnetic hyperfine interactions in the 4.2 K Mössbauer spectra of the siroheme and the iron-sulfur cluster demonstrates that each prosthetic group belongs to a system of half-integer electronic spin. This spin system must be common to both groups, because if both groups belonged to separate spin systems (one yielding the observed EPR signal, the other EPR silent), then an even number of electrons would be required to produce the diamagnetic state of SiR2−.CN, in contrast with the experiments (5) which show that SiR2−.CN is produced from SiR2+.CN by a 1-electron reduction.

In SiR3+, SiR2−, SiR2+, and SiR2−.NO the heme iron has unpaired d electrons and, thus, these states are suitable for the development of interatomic exchange interactions. In contrast, the complexes of SiR2− with CO and CN− as well as SiR2+ in 60% Me2SO have a low spin ferrous (S = 0) heme iron. In this state, the heme iron has a closed shell (t2g)6 configuration which is unfavorable for the development of exchange interactions. Therefore, these three complexes do not convey information, through the observation of magnetic effects, about the coupling of the chromophores.

Although we cannot prove that the two chromophores are directly linked by a bridging ligand in SiR3+.CO and SiR2−.CN, there is clear evidence that the two chromophores are interacting. First, the optical spectra of the heme show distinct changes when the iron-sulfur cluster is reduced from the 2+ to the 1+ state (5). Second, the redox potential of this step increases by 70 mV when CN− is replaced by CO (6). Furthermore, the quadrupole splitting of the low spin ferrous heme-CN complex changes when the iron-sulfur cluster is reduced. Finally, the magnetic hyperfine broadening observed for the [4Fe-4S]3+ cluster of SiR2+.CN suggests exchange-coupling of the prosthetic groups. The observations listed here suggest the maintenance of coupling but they do not prove it, because even if the chemical bond between the heme and the cluster were broken in the reduced CO and CN complexes, the chromophores would be expected to remain close enough for some interactive contact.

The [4Fe-4S] cluster has been observed here in two oxidation states. In SiR2−.CN and SiR2+.CN, it is in the 2+ state, whereas the 1+ state is observed for the 2-electron-reduced states of the SiR complexes. Our data show that the four iron sites of the [4Fe-4S]3+ cluster have, even within our good resolution, identical values for ΔE and δ. This is true in all states for which the SiR cluster is observed in the 2+ state. Furthermore, ΔE is independent of temperature for 1.5 K < T < 200 K. This temperature independence implies that there are no thermally accessible excited states with orbital character different from that of the ground state. In this respect, the SiR cluster is similar to the cluster of phosphoribosylpyrophosphate amidotransferase from Bacillus subtilis (25); all other clusters studied thus far exhibit temperature-dependent ΔE values. Our high field studies of SiR2−.CN
prove that the [4Fe-4S] cluster of SiR has a diamagnetic ground state in the 2+ state, in agreement with information obtained for [4Fe-4S] \textsuperscript{2+} clusters of other proteins and model complexes. This result demonstrates that the iron-sulfur cluster of SiR is intrinsically the same as the familiar clusters of other systems. This is significant in view of our observations that the electronic ground state of the cluster has acquired, through exchange-coupling to the siroheme, paramagnetism in some states of SiR, including SiR\textsuperscript{4+}-CN.

In the 2-electron-reduced states of SiR, the [4Fe-4S] cluster is observed in the 1+ state. Under conditions where the magnetic hyperfine interactions are averaged out, i.e. at higher temperatures in the limit of fast electronic spin relaxation, the 1+ states yield spectra consisting of two well resolved quadrupole doublets, each doublet representing two iron sites. The two types of sites exhibit quite different magnetic hyperfine interactions at 4.2 K. Site I has an isotropic A tensor with negative components whereas site II is characterized by an A tensor with substantial anisotropies and positive components. Since the iron sites of [4Fe-4S] clusters have a tetrahedral ligand coordination, they have high spin electronic configurations. A comparison of the parameters shows that site I has features reminiscent of ferric ions whereas site II has some ferrous character. This is borne out by the observation that site II exhibits the large values for \( \Delta E \) and \( A \), although the differences between the two sites are considerably less pronounced than the differences observed for the suitable model, ferric and ferrous rubredoxin (22, 27). The magnetic hyperfine interactions of high spin ferric ions derive from the isotropic Fermi contact term which is negative. In contrast, the A tensors of high spin ferrous sites are considerably anisotropic by virtue of large spin-dipolar and orbital contributions. The A values of sites I and II reflect this pattern. It is noteworthy that the "ferrous" site has positive A values; A tensors with positive components result when the local spin is oriented antiparallel to the system spin in a spin-coupled cluster (28, 29). In emphasizing that sites I and II are ferric and ferrous in character, respectively, we do not mean to imply that we are dealing here with trapped valences as observed for [2Fe-2S] ferredoxins. However, the degree of electron delocalization in reduced [4Fe-4S] clusters is not as pronounced as is often assumed in the literature.

We do not know whether the differences between sites I and II are primarily imposed by the environment of the iron-sulfur clusters. Recent studies of a centrosymmetric [2Fe-2S] model complex (30, 31) suggest that site inequivalencies can be an intrinsic electronic property of iron-sulfur clusters. Alternatively, one might attribute the differences observed here to the fact that the cluster is linked to the siroheme. This explanation is implausible in view of the fact that very similar Mössbauer parameters have been obtained for the ferredoxin from Bacillus stearothermophilus (14) and for a reconstituted [4Fe-4S] cluster in a ferredoxin from D. gigas (15). The capacity of [4Fe-4S] clusters to develop pronounced localized valences has recently been demonstrated for aconitase; upon binding of citrate, one site of the [4Fe-4S] cluster of that enzyme acquires a distinctly high spin ferrous character (32).

This paper together with our other studies of SiR (3, 4) provide the first Mössbauer results for Fe-isobacteriochlorin. Since the optical and EPR properties of siroheme (2, 5) and Fe-isobacteriochlorin model compounds (16) are quite different from those of porphyrins, it was of interest to see whether the Mössbauer spectra displayed unusual features attributable to the doubly reduced macrrole. Regarding the isomer shifts, we have observed no differences between the values for similar siroheme and porphyrin complexes. Values determined for the quadrupole splittings are also comparable, except for that of the ferrous siroheme-CO complex. The value of \( \Delta E \) for the latter is distinctly larger than those reported for porphyrin-CO complexes. However, until the ligand trans to the coordinated CO has been identified, it is not possible to decide whether this is attributable to the bacteriochlorin macrocycle. In the ferric ferric states, we have observed magnetic hyperfine interactions in addition to electric hyperfine interactions. An analysis (3) of the spectra of the high spin ferric siroheme yielded parameters which are quite typical for porphyrins. In fact, the values found for the zero-field splitting and the magnetic hyperfine coupling constant of the siroheme of SiR\textsuperscript{4+} match those reported for metmyoglobin (33, 34). The EPR results for the low spin Fe(III) siroheme-CN complex indicate ligand field parameters distinct from those of porphyrin-CN complexes. These differences seem to be reflected in the magnetic features of the Mössbauer spectra as well. (However, since the siroheme spectrum is partially masked by the contribution of the [4Fe-4S] cluster, we are prevented from making a thorough analysis of the siroheme spectrum.) Mössbauer studies of appropriate low spin ferric and low spin ferrous bacteriochlorin model complexes should be illuminating. However, one should keep in mind that the ligand trans to the CO and the CN- ligand possibly provides the link to the iron-sulfur cluster, therefore complicating interpretation of such model complex data.

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Mössbauer studies of Escherichia coli sulfite reductase complexes with carbon monoxide and cyanide. Exchange coupling and intrinsic properties of the [4Fe-4S] cluster.

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