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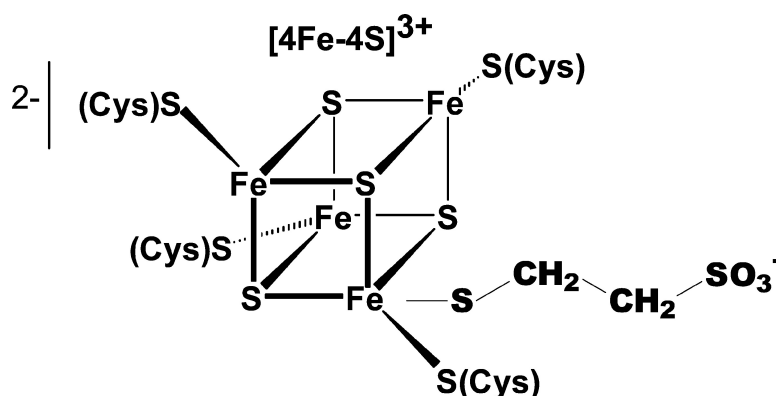
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# 57Fe ENDOR Spectroscopy on the Iron–Sulfur Cluster Involved in Substrate Reduction of Heterodisulfide Reductase

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Heterodisulfide reductase (Hdr) from methanogenic archea is an iron–sulfur protein that catalyzes the reversible two-electron reduction of the mixed disulfide CoM-S-S-CoB to the thiol coenzymes, coenzyme M (CoM-SH, 2-mercaptethane sulfonate) and coenzyme B (CoB-SH, 7-mercaptoheptanoylthreonine phosphate). In the final step of methanogenesis, the disulfide functions as the terminal electron acceptor for an energy-conserving electron transport chain, a process called disulfide respiration.[1] It is unusual that this enzyme uses an iron–sulfur cluster to mediate disulfide reduction in two one-electron steps via site-specific cluster chemistry. Recent studies reported the observation of a mechanistic-based paramagnetic intermediate generated upon half-reaction of the oxidized enzyme with CoM-SH in the absence of CoB-SH.[2] The $S = 1/2$ species, designated as CoM-Hdr, can be reduced in a one-electron step but not oxidized. The g-values, i.e., 2.013, 1.991, and 1.938 (for Hdr from *Methanothermobacter marburgensis*), and the EPR signal broadening in the ^{57}Fe-enriched enzyme[2] and ^{33}S-labeled CoM-SH,[4] in combination with variable-temperature magnetic circular dicroism (VTMCD) experiments,[3] lead to the proposal that CoM-Hdr is a novel substrate-bound [4Fe-4S]$^{3+}$ cluster with two thiolate ligands at a unique Fe site. However, direct evidence for the structure of this cluster is still elusive. In this contribution we employ ^{57}Fe pulsed ENDOR at two very different frequencies, 9 and 94 GHz, to identify the iron sites of CoM-Hdr and to provide more evidence for the ligation of the substrate to a unique iron site. We find direct evidence for a [4Fe-4S]$^{3+}$ cluster with unusual ^{57}Fe isotropic hyperfine coupling (hfc) values that reveal a complex nature of the interaction between the cluster and the CoM-SH substrate.

In Figure 1, we display ^{57}Fe pulsed ENDOR spectra of the intermediate CoM-Hdr recorded at the canonical orientations of the g-tensor in the EPR line. The 94 GHz spectra show a pattern of distinct absorptive and emissive lines, which can be assigned to doublets split by twice the ^{57}Fe Larmor frequency ν_L ($\nu_L = 4.8$ MHz at 3.5 T) and centered at half the value of the orientation-dependent hyperfine coupling A, according to $\nu_\pm \approx |A/2 \pm \nu_L|$, where $\nu_\pm$ is the position of the ENDOR line in first order. In contrast, the 9 GHz spectra are all absorptive and display doublets split by ∼0.96 MHz, according to the smaller Larmor frequency at 0.35 T. At all three field positions, the doublets at 9 GHz can be assigned to a pair of absorptive–emissive lines in the 94 GHz spectra following the above equation for $\nu_\pm$, as illustrated in Figure 1. Four doublets are detected at B||g₃ (Figure 1A), whereas at least three are resolved at B||g₁ and g₂ (Figure 1B,C). The simulations of the 9 GHz spectra (Figure 1), which take into account orientational selectivity[5] and ENDOR frequencies up to pseudo-first order,[6] are consistent with
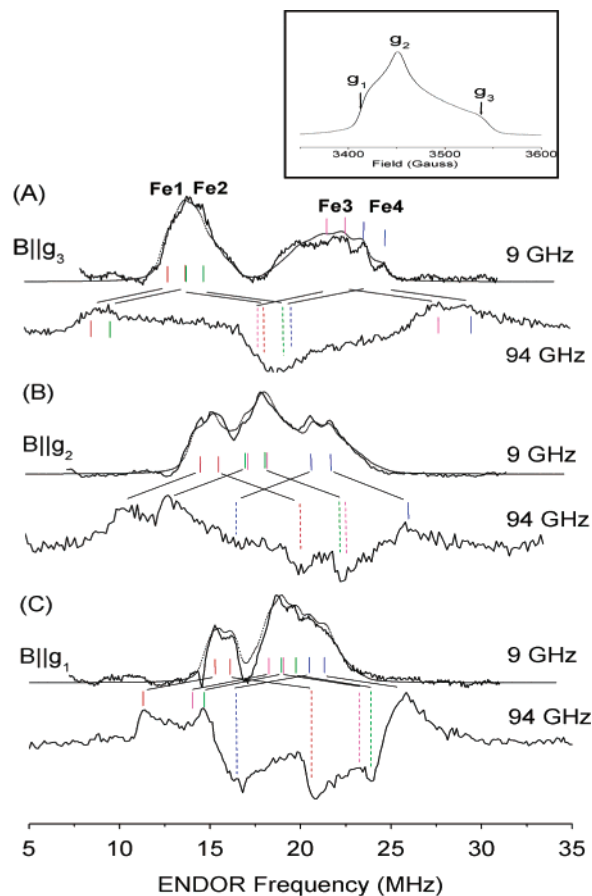
Figure 1. Davies ENDOR spectra of ^{57}Fe-enriched CoM-Hdr recorded at 9 and 94 GHz with Bruker spectrometers and at different positions of the EPR line according to B||g₃ (A), B||g₂ (B), and B||g₁ (C). Simulations are displayed as dotted lines. (Inset) Selected field orientations in the EPR line (9 GHz). Experimental conditions: $T = 4$ K; detection pulses ($\pi/2$), 100 ns (9 GHz) and 50 ns (94 GHz); preparation pulses, 50 ns (9 GHz) and 150 ns (94 GHz); RF pulses, 20−40 μs (9 GHz) and 55−100 μs (94 GHz); mixing time t_M (spacing between RF and detection pulses), 2 μs; repetition time, T_r, 100 ms; acquisition time, 5−12 h per spectrum. The 9 GHz spectra were recorded by strongly attenuating the overlapping ^{1}H resonances with a hard microwave preparation pulse.[8] The remaining features of the ^{1}H resonances were subtracted after a reference measurement (Supporting Information). CoM-Hdr was prepared from ^{57}Fe-enriched *M. marburgensis* Hdr and CoM-SH as described in ref 2.

four iron sites at each selected magnetic field position.[7] Simulations with three iron sites (not shown) unambiguously failed to reproduce the intensity ratios. The obtained hyperfine tensors are reported in Table 1. Thus, the data provide direct evidence for a [4Fe-4S] cluster in CoM-Hdr.

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Table 1. Simulated Principal Axis Values of the ^{57}Fe Hyperfine Tensors of the CoM-Hdr Paramagnetic Intermediate[a]

Fe site	A_x (MHz)	A_y (MHz)	A_z (MHz)	A_{iso} (MHz)
1	32.1	29.2	25.7	29
2	39.0	34.6	26.4	33.3
3	−37.2	−34.0	−46.6	−39.2
4	−39.7	−41.8	−48.7	−43.4

[a] Isotropic couplings are obtained as $A_{iso} = (A_x + A_y + A_z)/3$. The following nonzero Euler angles (in degrees) were found with respect to the g-tensor principal axis: (Fe1) $\beta = 10$, (Fe2) $\alpha = 10$, (Fe3) $\beta = 31$, (Fe4) $\beta = 30$, $\gamma = 40$.

The observation of highly polarized patterns in W-band ENDOR permits determination of the sign of the hfc's,[9,10] a new advantage of high-field ENDOR that leads to useful information for the assignment of the cluster. We note that the polarization effect can be qualitatively described in a simple "hole burning" picture, well known in optical spectroscopy, where selective pumping on one of the two ENDOR transitions associated to a $I = \frac{1}{2}$ nucleus creates holes or anti-holes outside the microwave excitation range, provided nuclear spin relaxation is slow compared to the pulse repetition rate. Following the model of ref 10, at our experimental conditions (t_M, $T_{1e} < T_r$, positive nuclear g-factor) and under the assumption of a very long T_{1N},[11] a positive hfc leads to an absorptive ν_- (associated with $m_s = \frac{1}{2}$) and an emissive ν_+ ($m_s = -\frac{1}{2}$) and vice versa for a negative hfc (see also Supporting Information). Thus, the sign of the hfc associated with Fe sites 1 and 2 is positive, whereas the sign of sites 3 and 4 is negative. The result is most consistent with the sign of the hfc's found in [4Fe-4S]$^{3+,+}$ clusters by Mössbauer spectroscopy, where the iron pair with the largest hfc's (Fe$^{2.5+}$−Fe$^{2.5+}$) has negative sign, while those containing either the ferrous or the ferric pair are positive and of smaller magnitude.[12,13]

Once it is established that CoM-Hdr contains a [4Fe-4S] cluster, the observation of this paramagnetic species only under oxidizing conditions[2] strongly indicates that the cluster occurs in the oxidized 3+ state, in accordance with the signs of the hfc's and the VTMCD experiments. Comparing the results with ^{57}Fe hfc's in [4Fe-4S]$^{3+}$ clusters reported from ENDOR of model complexes[14] and HiPIP proteins,[15,16] we find typical isotropic hfc's of the mixed-valence pair around −30 MHz and of the ferric (Fe^{3+}−Fe^{3+}) pair around 20 MHz, values which are similar to but systematically lower than those found in CoM-Hdr ($|31| < A_{iso} < |44|$ MHz). Thus, the results suggest that the enhanced couplings observed here are due to the interaction of the cluster with the substrate.

Very recently, evidence for a five-fold-coordinated [4Fe-4S]$^{3+}$ cluster was provided in an NEM-alkylated form (NEM-FTR) of ferredoxin:thioredoxin reductase (FTR), an enzyme which—similarly to Hdr—catalyzes a disulfide cleavage in two one-electron steps involving [4Fe-4S] chemistry.[17] In NEM-FTR, a cysteine derived from the active-site disulfide ligates to a unique Fe site of the active-site [4Fe-4S]$^{3+}$ cluster.[18,19] The reported[17,19] ^{57}Fe hfc's ($A_{iso} = +22$ and $+27$ MHz for the ferric and $−37$ MHz for the mixed-valenced pair) show—similarly to CoM-Hdr—some inequivalency and slightly enhanced hfc values with respect to the [4Fe-4S]$^{3+}$ HiPIP clusters and model systems. The parallel trends of the hfc's in NEM-FTR and CoM-Hdr could be indicative of a five-fold coordination at a unique iron site in CoM-Hdr, supporting the proposal from the EPR[4] and VTMCD data.[3] Nevertheless, despite the similarities, in NEM-FTR a pronounced anisotropy (~66%) of the ^{57}Fe hfc was reported at the unique site[19] which is not observed here. A compelling difference between the clusters in NEM-FTR and CoM-Hdr was also noted in the g-values,[3] with $g_{av} > 2.0$ for NEM-FTR, as is typical for [4Fe-4S]$^{3+}$ clusters,

whereas $g_{av} < 2.0$ for CoM-Hdr is intriguing. Furthermore, the cysteines that ligate the active-site [4Fe-4S] cluster in FTR[18] are not conserved in Hdr. Here the active-site [4Fe-4S] cluster is proposed to be ligated by cysteines of a CX$_{31−32}$CCX$_{33−38}$CX$_2$C motif of which the enzyme contains two copies.[1] Hence, these observations suggest some unique properties of CoM-Hdr.

Recently, several studies of SAM-dependent Fe−S enzymes have underlined the new role of [4Fe-4S] clusters involved in the chemistry of the substrate and have demonstrated that the [4Fe-4S] cluster and the substrate (AdoMet) interact at multiple sites.[20] For CoM-Hdr, while it has been established that the thiolate group of CoM-SH binds to an iron of the cluster,[4] the role of the large, negatively charged sulfonate group of CoM-SH is still unclear. As the substrate is flexible, the interaction could involve coordination at more than one site. ENDOR experiments with ^{17}O- and ^{2}H-labeled substrates are underway to clarify this point.

Supporting Information Available: Reference 9 GHz spectra, W-band simulations, and the sign of the hfcs from the 94 GHz spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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