

MÖSSBAUER SPECTROSCOPY APPLIED TO THE OXIDIZED AND SEMI-REDUCED
STATES OF THE IRON-MOLYBDENUM COFACTOR OF NITROGENASE

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Mössbauer parameters at 125K for both the oxidized and semi-reduced states of FeMoco isolated from the MoFe protein of *Azotobacter vinelandii* nitrogenase of $\delta/Fe=0.32$ and 0.37 mm/s and $\Delta E_Q=0.84$ and 0.71 mm/s, respectively, are reported. FeMoco(ox) fits the Debye model perfectly from 4.2-125K and has a S=0 ground state. FeMoco(ox) apparently contains 10-20% FeMoco(s-r) and *vice versa*, possibly as a result of the spontaneous oxidation phenomenon. Quantitation of the spectra indicates a Fe:Mo ratio of 5±1:1 and the similar quadrupole splittings and isomer shifts suggest a similar environment for all iron atoms. © 1989 Academic Press, Inc.

FeMoco extruded from the MoFe protein of nitrogenase has been under active study with respect to its molecular structure, particularly through XAS (1-5), its composition (6-8), and oxidation-reduction properties (9, 10), since its original isolation (11). Isolated FeMoco retains its characteristic spectroscopic signatures, only slightly changed from those observed when bound within the protein (1,2,12,13), and is capable of restoring dinitrogen-fixing activity to the FeMoco-deficient, inactive nitrogenase of various mutants of *Azotobacter vinelandii* (11,14) and *Klebsiella pneumoniae* (15). Genetic evidence strongly implies that FeMoco is, contains, or is part of the N₂-binding site (16). Thus, studies of this simpler entity should give insight into the mechanism of biological dinitrogen reduction.

Although thermally stable in non-aqueous solvents (6,11,17), FeMoco has resisted all attempts to produce X-ray diffraction quality crystals. However, XAS at the Mo K-edge shows three iron, three sulfur and three oxygen nearest neighbors (1,2). How the remaining atoms are arranged and exactly how many

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Abbreviations Used: FeMoco, iron-molybdenum cofactor; MoFe protein, molybdenum-iron protein of nitrogenase; XAS, X-ray absorption spectroscopy; NMF, N-methylformamide; EPR, electron paramagnetic resonance; (ox), oxidized; (s-r), semi-reduced; (red), reduced; ENDOR, electron-nuclear double resonance; EFG, electric field gradient.

of each are required to complete the FeMoco unit are unknown. FeMoco in NMF exhibits two redox couples, which interconvert the EPR-active $S = 3/2$ FeMoco(s-r), the state produced by excess dithionite, with both a more oxidized state, FeMoco(ox), and a more reduced state, FeMoco(red) (9). The (ox)-to-(s-r) change is a reversible, one-electron process (9, 10).

In contrast to the intensive Mössbauer (18-20) investigation of the holo-MoFe protein, much of which impacts directly on FeMoco bound within the protein, one report only mentions the Mössbauer spectrum of isolated FeMoco(s-r) (12). The lack of comparative information for bound versus isolated FeMoco in both (ox) and (s-r) states, plus our desire to: (i) quantitate the Fe content of FeMoco; (ii) determine the spin state of FeMoco(ox); (iii) determine where the added e^- is accommodated in FeMoco(s-r); and (iv) gain insight into the symmetry of this heterometallic cluster, led to the Mössbauer study reported herein, which emphasizes the FeMoco(ox) state.

MATERIALS AND METHODS

The Mössbauer spectrometer used has been described previously (21,22). It permits very precise measurement of the source lineshape (21), which allows us to deconvolve the data and perform the logarithmic conversion from transmission to absorption spectra (22,23). However, it is not possible to present simple absorption spectra, when there are non-trivial magnetic terms present in the spin Hamiltonian (21). Therefore, because some spectra are taken under high applied magnetic fields, we show all spectra in transmission mode for consistency.

^{57}Fe -labelled FeMoco was isolated from *Azotobacter vinelandii* strain OP, which was grown in batch culture in acid-washed glass vessels essentially as described previously (24) except that the Burk nitrogen-free medium contained ^{57}Fe (New England Nuclear, 96.5%) at only 20% of the usual iron concentration. Isolation and analysis of the ^{57}Fe -labelled nitrogenase proteins were as reported earlier (24). The ^{57}Fe -enriched MoFe protein of specific activity 1877 nmol C_2H_4 formed min^{-1} (mg protein) $^{-1}$ contained 24.5 iron atoms per molecule (25).

^{57}Fe -enriched FeMoco was released from this protein into NMF and concentrated as previously described (6). Its specific activity was 197 nmol C_2H_4 formed min^{-1} (ng. atom Mo) $^{-1}$. The Mo and Fe concentrations were 0.214 mM and 1.055 mM, respectively, (25,26) giving Fe:Mo of 4.9:1. Aliquots were transferred to a Mössbauer cuvette (200 μL) and an EPR tube (300 μL) in a Vacuum Atmospheres anaerobic glove box, sealed, removed from the glove box and frozen in liquid N_2 . EPR spectroscopy (19) at 10K showed a $S=3/2$ signal with g values typical of FeMoco(s-r). Mössbauer spectroscopy (see Results and Discussion section) indicated some FeMoco(ox), so the sample was thawed anaerobically, dithionite (10 μL of 0.2M solution) added, and, after 10 min, refrozen in liquid N_2 . The remainder of the sample was allowed to oxidize spontaneously (9) and anaerobically at -80°C over a period of four weeks. Mössbauer and EPR aliquots were then removed and frozen as before. Unenriched 0.734 and 1.054 mM (in Mo) FeMoco samples in both the (ox) and (s-r) states were prepared and investigated similarly. The Fe:Mo ratio for both samples was 5.8:1 with specific activities of 170 nmol C_2H_4 formed min^{-1} (ng. atom Mo) $^{-1}$. The EPR samples of FeMoco(ox) were EPR-silent. EPR experiments were also performed directly on the samples in the Mössbauer cuvettes using a Varian "wide mouth" cylindrical cavity operating in the TE-013 mode. The FeMoco(s-r) samples gave the expected $S = 3/2$ EPR spectrum, but the experiments failed to detect the

FeMoco(s-r) molecules in the FeMoco(ox) Mössbauer samples because the low filling factor of the cavity generated insufficient signal-to-noise.

RESULTS AND DISCUSSION

Fig. 1 shows a stackplot of FeMoco(ox) at zero applied magnetic field and at temperatures from 4.2 to 175K. A major quadrupole doublet is visible at all temperatures. In addition, three other signals are observed. The first, a high-spin ferrous component, has been observed previously (12). Its

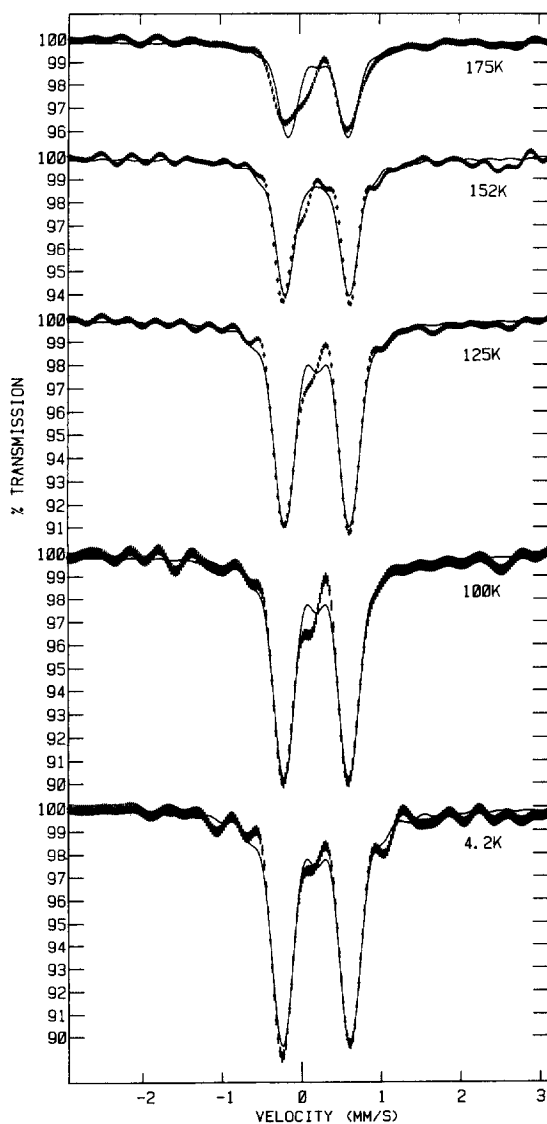


Figure 1. Mössbauer spectra of ^{57}Fe -enriched FeMoco(ox) in zero applied magnetic field as a function of temperature. The solid lines are single site fits with the parameters shown in Table 1.

right-hand line is sometimes visible at 2.6 mm/s. The second signal is the quadrupole doublet ($\delta/\text{Fe} = 0.55$ mm/s and $\Delta\text{Eq} = 0.88$ mm/s) that appears as shoulders on the right-hand side of the major doublet. We have very carefully quantitated these signals as maximally 3% and 6% of total iron, respectively; much less than the 12.5% of intensity minimally expected for an integral component of intact FeMoco. Both then are assigned to low-concentration contaminants. The third signal (~13%) arises from FeMoco(s-r). Its concentration was estimated from the decrease in intensity of the main quadrupole doublet as temperature is lowered (Table 1), because the FeMoco(s-r) quadrupole doublet lies within the lineshape of FeMoco(ox).

The amounts of iron listed in Table 1 include an assumed Debye temperature of 188K, which was chosen to perfect the model between 100 and 125K. If the sum of the intensity of all the lines is used, then the fit of the Debye model to intensity is perfect over 4.2-125K. There is, however, a very narrow temperature range, near 125K, where the FeMoco(ox) resonances are faithfully represented by quadruple doublets. Above 150K, the intensity decreases due to the onset of the liquid state of NMF. The individual iron atoms of isolated FeMoco, therefore, do not have Debye-Waller factors with different temperature dependencies as reported for the 'P' clusters of the MoFe protein (19). The isomer shift variation with temperature (table 1) is probably due to the 2nd-order relativistic Doppler shift, but no such correction is applied. A similar temperature dependence of isomer shift is found with spectra of both model compounds (prismanes and cubanes) and nitrogenase.

Table 1. Best Fit Parameters of the Two-Line Model for FeMoco(ox)^{a,b}

T (K)	δ/Fe (mm/s)	ΔEq (mm/s)	# $\mu\text{moles } ^{57}\text{Fe}$
175	0.32	0.76	0.12
152	0.33	0.81	0.21
125	0.33	0.82	0.22
100	0.34	0.82	0.22
4.2	0.35	0.85	0.19

^aUncertainty values are 0.01, 0.02 and 0.01 for δ/Fe , ΔEq and # $\mu\text{moles } ^{57}\text{Fe}$, respectively (22).

^bBecause these spectra result from centers with ca. six iron atoms, these single site fitting parameters represent average values that can be modeled under the simulation linewidth of 0.27 mm/s (FWHM).

To determine if FeMoco(ox) is diamagnetic at low temperature (Fig. 2), a very intense magnetic field (5 Tesla) was applied to the sample at 4.2 K to ensure that the expected Zeeman splitting of an integer spin system would be greater than kT and the expected zero-field splittings. For non-zero spin, magnetic splitting from the ground state of the spin multiplet is expected, but the only magnetic splitting observed is due to the minor contribution from FeMoco(s-r). This result proves that isolated FeMoco(ox) has a $S=0$ ground state, the same as when bound to the protein (27).

The spectra of FeMoco(s-r) at high temperature and at low temperature and low applied field are shown in Figure 3. Under each set of conditions, two spectra are reported. The initial point spectra indicated the presence of FeMoco(ox), which was subsequently reduced by added dithionite as shown by the diminished intensity of the central quadrupole doublet and the increased intensity of the outer lines. Based on estimated intensity of the central

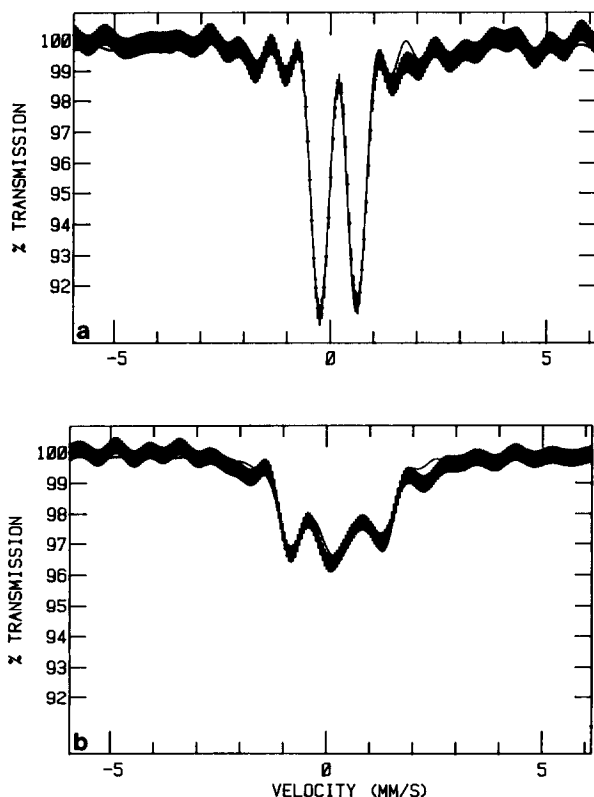


Figure 2. Low temperature (4.2K) Mössbauer spectra of ^{57}Fe -enriched FeMoco(ox) at 0.06 Tesla (a) and at 5 Tesla (b) applied magnetic field. The solid lines are computer simulations with $\Delta E_q = 0.85$ mm/s, asymmetry parameter $\eta = 0$, the appropriate applied fields and with linewidths of 0.27 mm/s (a) or 0.45 mm/s (b).

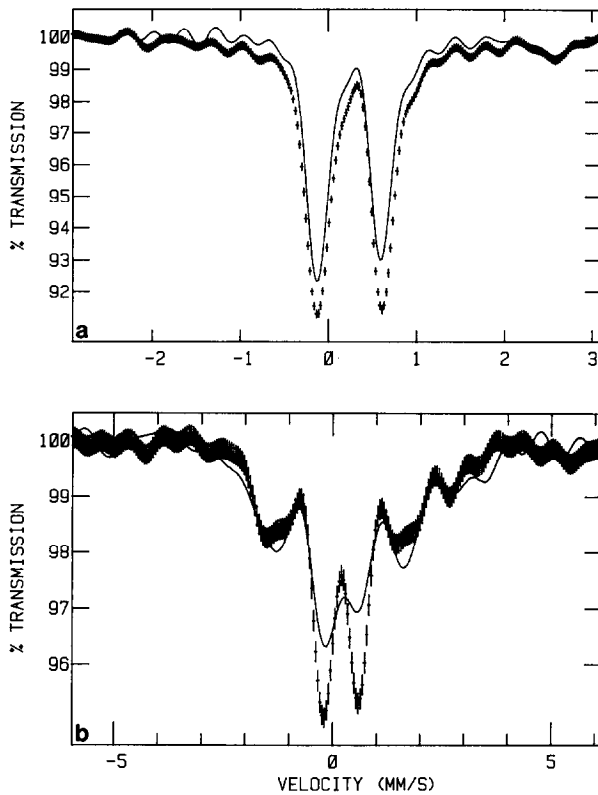


Figure 3. Mössbauer spectra of ^{57}Fe -enriched FeMoco(s-r) at 125K in the absence of an applied field (a) and at 4.2K in a 0.06 Tesla applied magnetic field (b); (.....) initial spectra, (—) after additional aqueous dithionite. The solid line spectra have slightly reduced intensity due to dilution.

quadrupole pair, a change from ca. 30% oxidized to ca. 20% oxidized occurred, assuming identical Debye temperatures for FeMoco(ox) and (s-r). These low-temperature spectra are entirely consistent with that reported previously (12), which also contained ca. 20% of its intensity as a then uncharacterized quadrupole doublet that we now suggest is FeMoco(ox). Thus, the spectra of isolated FeMoco(ox) appear to contain a contribution from FeMoco(s-r) and vice versa, possibly due to the spontaneous oxidation phenomenon (9,28). If this situation is germane to the MoFe protein, then interpretation of the Mössbauer spectra of many protein samples could be severely compromised.

By using the low temperature spectra to quantitate the percent reduction of the sample, the best fit of the high temperature components in Figs. 1c and 3a can be calculated after subtraction of the minor component. Iteration of this fitting procedure gives the parameters for pure FeMoco(ox) and (s-r) (Table 2), which show that the change in isomer shift accompanying a one-elec-

Table 2. Mössbauer Parameters of FeMoco and Chemical Models

Compound	T (K)	δ/Fe (mm/s)	ΔEq (mm/s)	μmoles ^{57}Fe	Ref.
FeMoco(ox) ^a	125	0.32	0.84	0.22	This work
FeMoco(s-r) ^a	125	0.37	0.71	0.17	This work
FeMoco(s-r)	90	0.37	0.75	----	(12)
(Ph ₄ P) ₂ Fe ₆ S ₆ Cl ₆	125	0.43	0.62	----	(34)
(Et ₄ N) ₃ Fe ₆ S ₆ Cl ₆	125	0.50	1.09	----	(35)
(Et ₄ N) ₂ Fe ₄ S ₄ (SCH ₂ Ph) ₄	77	0.36	1.10	----	(36)
(Et ₄ N) ₃ Fe ₄ S ₄ (SCH ₂ Ph) ₄	77	0.44	1.00	----	(37)
(Bu ₄ N) ₃ Mo ₂ Fe ₆ S ₈ (SPh) ₉	130	0.28 ^b	1.12 ^b	----	(38)
(Et ₄ N) ₅ Mo ₂ Fe ₆ S ₈ (SPh) ₉	130	0.39 ^b	1.28 ^b	----	(38)

^aThese measurements and the related simulations assume that both lines of the quadrupole pairs have a linewidth of 0.27 mm/s (FWHM).

^bAverage of parameters for two inequivalent sites.

tron change is 0.05 mm/s. The analogous shift for cubane and prismane models is about 0.08 mm/s ($p = 0.01$). As all are iron/sulfur clusters with similar numbers of iron atoms, the reducing electron appears to be delocalized less onto the iron atoms of FeMoco. Certainly, orbitals with a large molybdenum component are good candidates for accommodating the additional charge density but recent XAS work is inconsistent with this suggestion, leaving orbitals with a large sulfur component as the additional acceptors (3,30).

An accurate determination of FeMoco's Fe-to-Mo ratio is needed. Because our spectrometer is quantitative, iron content is easily determined which, with the Mo content determined chemically, indicates a $5.0 \pm 0.5:1$ ratio for the ^{57}Fe -enriched samples. Four other FeMoco samples isolated with naturally abundant ^{57}Fe contents had a ratio of $5.25 \pm 0.25:1$. All contain ca. 9% of total iron as impurities. Assuming only iron in the main doublet is in FeMoco, plus all the molybdenum, a Fe:Mo ratio of 4.3:1 results. If the minor quadrupole doublet, which is responsible for the 6% iron impurity, also contains molybdenum in a 1:1 ratio, the Fe:Mo ratio approximates 6:1. Obviously, the Fe:Mo ratio depends very strongly on both Mo content and Fe-containing impurities and can only be approximated as $5 \pm 1:1$. This value encompasses a previous estimate (12). The accuracy of Mössbauer spectroscopic iron determinations depends critically on the validity of the model

used. For the MoFe protein (18), the intensity of a particular spectral line is used to quantitate others. The complexity of the spectra shown herein hint that this spectroscopic model may lack the accuracy necessary to determine FeMoco's Fe:Mo ratio. ENDOR measurements (31) are free of some of these interpretation difficulties but unfortunately, this technique has not yet been applied to isolated FeMoco. Even so, the five distinct ^{57}Fe resonances reported for bound FeMoco(s-r) confirm a Fe:Mo ratio of at least 5:1.

The narrow linewidths of our quadrupole doublets suggest equivalent environments for iron in isolated FeMoco. In contrast, based on an analysis of the ENDOR ^{57}Fe magnetic hyperfine tensor components, low symmetry is suggested for bound FeMoco (31). However, the ENDOR work ignored the importance in spin coupled systems of the projection operator term (32, 33), which describes the orientation of component spins relative to the resultant. For bound FeMoco, three iron atoms have negative and two have positive hyperfine couplings (31), indicating complex spin coupling among an unknown number of components. This situation makes it impossible to determine symmetry by comparing the magnitude of hyperfine coupling tensors. However, the single site fit to our data (Fig. 2b) implies that the EFG tensors could be identical for all FeMoco iron atoms. To the extent that the EFG tensor connotes ligand symmetry, such identity is a strong constraint on site geometry - much stronger than spin-coupling details implied by the magnitude of hyperfine coupling tensors. Further, the quadrupole doublet in the 4.2K spectrum of FeMoco(s-r) (Fig. 3b) could be due to an iron site of FeMoco(s-r) with the projection operator term equal to zero rather than to residual FeMoco(ox). In this case, the Fe-to-Mo ratio of FeMoco would increase to 6+1:1. We would, therefore, point the FeMoco model builders toward a structure that is symmetric with respect to the iron atoms (at least five and possibly six) and oxidized with respect to the irons in the oxidized (2-) prismane.

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