7.8: Multisite Redox Enzymes (Part 4)

7. EPR, ENDOR, and ESEEM Studies

The FeMoco or M center has been identified spectroscopically within the FeMo protein;[239,248,273] it has a distinctive EPR signal with effective g values of 4.3, 3.7, and 2.01, and originates from an S = $\frac{3}{2}$ state of the M center. The signal arises from transitions within the $\pm\frac{1}{2}$ ground-state Kramers doublet of the S = $\frac{3}{2}$ system (D = +5.1 cm^{-1}, E/D = 0.04). The isolated cofactor (FeMoco) gives a similar EPR signal, but with a rather larger rhombicity (E/D = 0.12). Spectra from the *C. pasteurianum* nitrogenase and cofactor are shown in Figure 7.28, and comparative data are given in Table 7.7. The M-center EPR signal has proved useful in characterizing the nature of the site, especially when more sophisticated magnetic resonance techniques, such as ENDOR or ESEEM, are used.

Extensive ENDOR investigations[274,275,275a] have been reported using protein samples enriched with the stable magnetic isotopes ^{2}H, ^{33}S, ^{57}Fe, ^{95}Mo, and ^{97}Mo. The ^{57}Fe couplings have been investigated in the most detail. Individual hyperfine tensors of five coupled ^{57}Fe nuclei are discernible, and were evaluated by simulation of the polycrystalline ENDOR spectrum.[275] The data from ^{33}S and ^{95}Mo were analyzed in less detail; ^{33}S gave a complex ENDOR spectrum, evidently with quite large hyperfine couplings, although no quantification was attempted because of the complexity of the spectrum.[274] On the other hand, ^{95}Mo was shown to possess a small hyperfine coupling, indicating that the molybdenum possesses very little spin density (although the quantitative aspects of the conclusions of the ^{95}Mo ENDOR study have recently been shown to be in error[276]).

Although no nitrogen splittings were reported in any of the ENDOR studies, evidence for involvement of nitrogen as a cluster component has been forthcoming from ESEEM spectroscopy.[277-279] ^{14}N modulations are observed in the ESEEM of the M center. The observed ^{14}N is not from the substrate (N_2), or from an intermediate or product of nitrogen fixation, because enzyme turnover using ^{15}N as a substrate does not change the ESEEM spectrum. The isolated cofactor (FeMoco) does not show the modulation frequencies observed for the M center in the protein. These experiments suggest that the M-center ^{14}N ESEEM arises from a nitrogen atom that is associated with the M center, and probably from an amino-acid side chain (most likely a histidine) ligated to the cluster.[279] Recent evidence from site-directed mutagenesis of the *Azotobacter vinelandii* protein[280] provides strong support for the presence of histidine ligation, and points specifically to His-195 of the α subunit as the N ligand.

8. Mössbauer Studies

Extensive Mossbauer investigations of nitrogenase[271,281-283] and FeMoco[283a] have been reported. Unlike EPR and EPR-based spectroscopies, which can be used to investigate only the EPR-active S = $\frac{3}{2}$ oxidation state, all three available M-center oxidation states are accessible to Mössbauer spectroscopy. The fully reduced site was found to be diamagnetic with S = 0 (but see Reference 284), whereas the oxidized site was found to have S $\geq$ 1. The zero-field spectrum of reduced *C. pasteurianum* nitrogenase is shown in Figure 7.29; the spectrum is comprised of four quadrupole doublets, one of which was concluded to originate from the M site.[282] Mössbauer spectra taken in the presence of applied magnetic fields were used to deduce the presence of four types of ^{57}Fe hyperfine coupling; these were called sites A1, A2, and A3, which have negative hyperfine couplings, and B sites, which have positive hyperfine couplings. The A sites were quantitated as a single Fe each; the B sites were estimated to contain three irons. These conclusions were largely confirmed and extended by later ENDOR investigations,[274] although the B sites were resolved as two inequivalent, rather than three equivalent, sites. ENDOR is rather more sensitive to the nature of the hyperfine couplings than Mössbauer, although it cannot usually be used to count numbers of exactly equivalent sites. Thus the number of iron atoms in the M center is minimally five, although larger numbers cannot be excluded. Note also that some of the quantitative aspects of the earlier Mossbauer investigations have been criticized.[285]

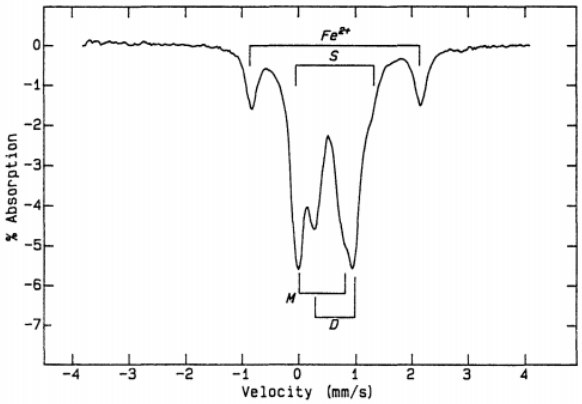
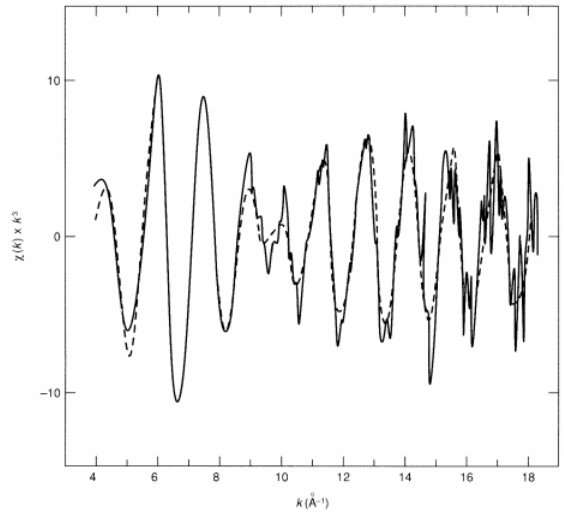


Figure 7.29 - Mössbauer spectrum of C. pasteurianum nitrogenase FeMo protein,[282] indicating the various components (quadrupole doublets) and their assignments. The doublet labeled M is the cofactor signal; those labeled D, S, and Fe^{2+} are attributed to the P-clusters.

9. X-ray Absorption Studies

One of the early triumphs of biological x-ray absorption spectroscopy was the deduction that the nitrogenase M center is an Mo-Fe-S cluster.[286] (It is also worth noting that nitrogenase was the first *enzyme* to be studied by x-ray absorption spectroscopy.) Early work on lyophilized protein samples indicated the presence of two major contributions to the Mo K-edge EXAFS, which were attributed to Mo-S ligands, plus a more distant Mo-Fe contribution.[286] Subsequently, these conclusions have been confirmed and extended, using samples in solution and with much more sensitive detection systems.

Most EXAFS studies to date have been on the molybdenum K-edge of the protein or of FeMoco, and indicate a very similar Mo environment in both (Table 7.7, Figure 7.30). A consensus of the best available analyses[287] indicates that Mo is coordinated by three or four sulfur atoms at 2.4 Å, one to three oxygens or nitrogens at 2.2 Å, with approximately three nearby iron atoms at 2.7 Å. Of these, the EXAFS evidence for the oxygen/nitrogen contribution is weakest. However, comparison of Mo K-edge[288] and Mo L-edge XANES[289] spectra with model compounds indicates strong similarities with $MoFe_3S_4$ thiocubane model compounds possessing MoS_3O_3 coordination, and provides some support for the presence of O/N ligands.



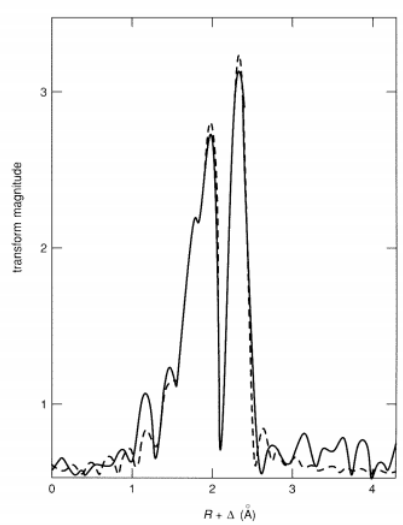


Figure 7.30 - Mo K-edge EXAFS spectrum (left panel) and EXAFS Fourier transform (right panel) of Klebsiella pneumoniae nitrogenase MoFe protein. The solid line is the processed experimental spectrum and the dashed line a calculated one. [287]

The iron EXAFS of FeMoco has been independently examined by two groups.[290,291] Both groups agree that the iron is coordinated largely to sulfur at about 2.2 Å, with more distant Fe-Fe interactions at about 2.6 Å. They differ, however, concerning the presence of short (1.8 Å) Fe-O interactions. Such interactions were apparently observed in the earlier study,[290] but not in the later study.[291] One possible explanation for this discrepancy is that the short Fe-O interactions of the earlier study were due to extraneous iron coordinated to solvent, contaminating the FeMoco preparation.[291] A final resolution of this discord must, however, await the results of further experiments. Interestingly, a long Fe-Fe interaction at 3.7 Å was also observed in the later study.[291]

Largely on the basis of the Mo K-edge EXAFS results and model studies discussed below, several proposals for the structure of the M center have been put forward. These are illustrated in Figure 7.31.

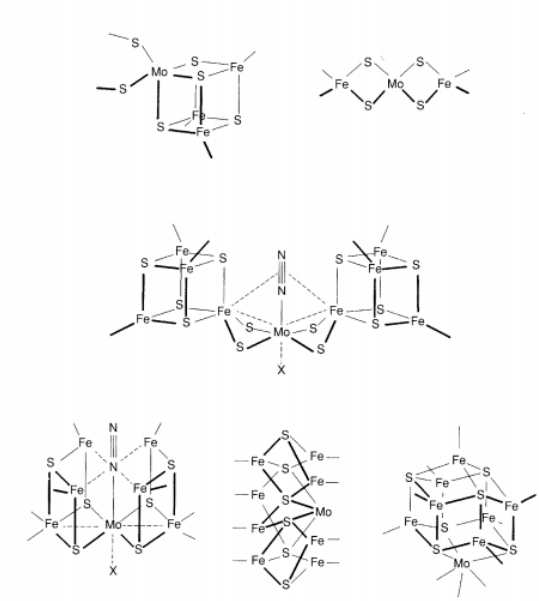


Figure 7.31 - Proposed models for FeMoco. (Compare with the recent model from the x-ray structure on page 444.)

The MoFe proteins from *Clostridium pasteurianum*[292] and from *Azotobacter vinelandii*[293] have been crystallized. For the former protein, crystals of space group $P2_1$ are obtained, with two molecules per unit cell of dimensions 70 x 151 x 122 Å. There is good evidence for a molecular two-fold axis, which presumably relates equivalent sites in the two $\alpha\beta$ dimers that make up the protein molecule.[294] Preliminary refinement reveals that the two FeMoco units per protein are about 70 Å apart and the four P clusters are grouped in two pairs.

Single crystal EXAFS studies[295] have provided important structural information on the molybdenum site. For different crystal orientations (relative to the polarized x-ray beam), the amplitude of the Mo-Fe EXAFS changes by a factor of 2.5, but the Mo-S EXAFS changes only slightly. Analysis of the anisotropy of the Mo-Fe EXAFS using the available crystallographic information[294] is consistent with either a tetrahedral $MoFe_3$ geometry such as that found in thiocubanes (Figure 7.32) or a square-based pyramidal $MoFe_4$ arrangement of metals. This interpretation tends to rule out some of the structural proposals shown in Figure 7.33. The observed orientation-dependence of the iron amplitudes is too small for clusters containing a linear or planar arrangement of iron and molybdenum (e.g., Figure 7.33B,C), and too large for arrangements that involve regular disposition of iron about molybdenum. Moreover, the lack of anisotropy of the sulfur EXAFS (which was apparently not considered in the original interpretation[295]) argues against an $MoS_3(O/N)_3$ model that has molybdenum coordinated by sulfur atoms that bridge only to Fe atoms disposed to one side of the molybdenum. Significant anisotropy for the Mo-S EXAFS (of opposite polarization, and smaller than that for Mo-Fe) would be expected for such an arrangement of sulfur atoms. However, the cubane model of Figure 7.33, which provides the best model of both geometric and electronic structure, remains viable if one of the nonbridging ligands to molybdenum is a sulfur atom (rather than oxygen or nitrogen) with a bond length similar to that of the bridging sulfides.

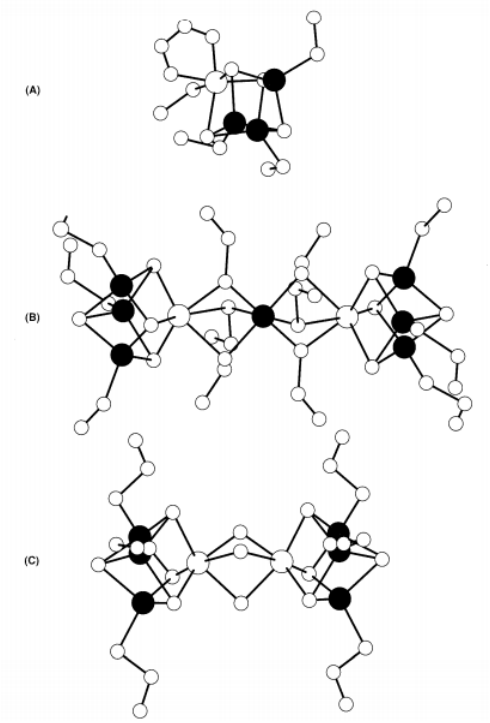


Figure 7.32 - Structures of thiocubanes that display Mo-S and Mo-Fe distances similar to FeMoco: (A) $(Fe_3MoS_4)_2(SR)_9^{3-}$; (B) $(MoFe_3S_4)_2Fe(SR)_{12}^{3-/4-}$; (C) $MoFe_3S_4(SEt)_3(cat)CN^{3-}$. (Data on A and B from References 328, 330a; data on C from References 331, 332.)

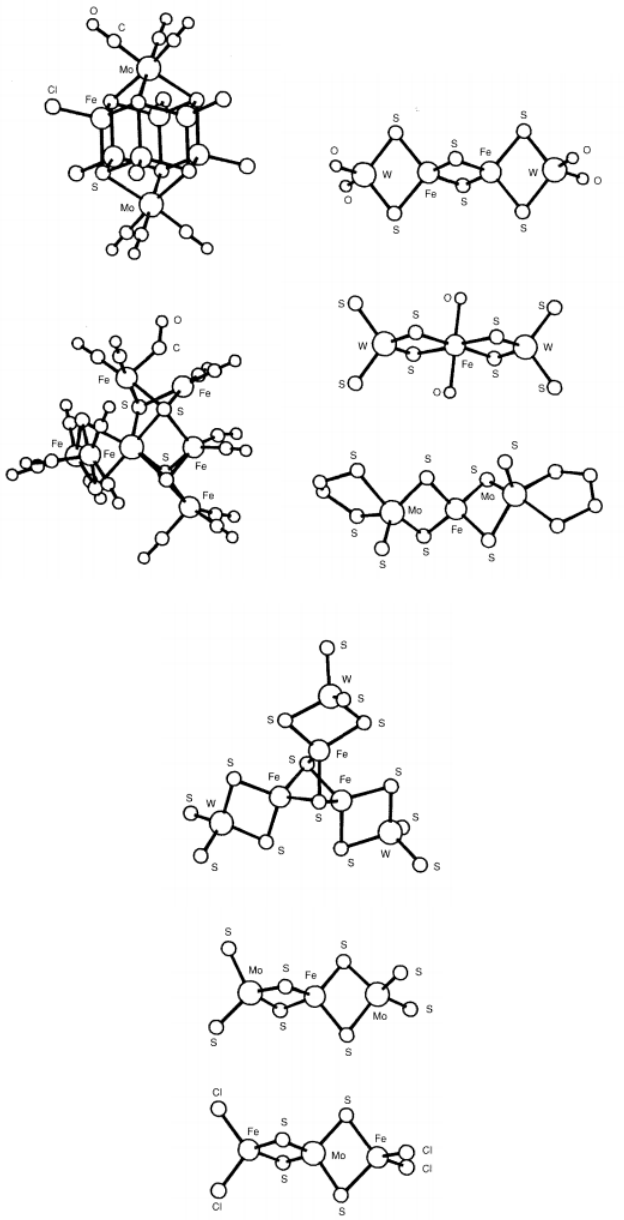


Figure 7.33 - FeMoS and FeWS structures of potential interest with respect to nitrogenase.[331,332,332a-j]

10. Substrate Reactions

The two-component Mo-nitrogenase enzyme catalyzes the reduction of N_2 to $2NH_4^+$ as its physiological reaction. Concomitant with the reduction of N_2, H_2 evolution occurs, with electrons supplied by the same reductants that reduce N_2. The limiting stoichiometry appears to be

$$N_2 + 10H^+ + 8E^- \rightarrow 2NH_4^+ + H_2 \tag{7.16}$$

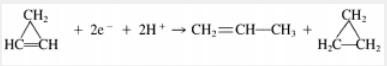
If N_2 is omitted from the assay, all the electrons go to H_2 evolution. Indeed, to a first approximation the rate of electron flow through nitrogenase is independent of whether the enzyme is producing only H_2, producing both NH_4^+ and H_2, or reducing most of the alternative substrates. As displayed in Table 7.8, many alternative substrates are known for this enzyme.[240,243,296] The most important of these from a practical perspective is acetylene, C_2H_2, which is reduced by the Mo nitrogenase exclusively to ethylene, C_2H_4. Acetylene can completely eliminate H_2 evolution by nitrogenase. Many of the substrates in Table 7.8 have a triple bond. Indeed, the only triple-bonded molecule not reduced by nitrogenase is CO, which nevertheless inhibits all substrate reactions, but not H_2 evolution (in the wild type). Triple-bonded molecules such as acetylene (H—C≡C—H) are useful probe molecules for related reactivity as discussed below for simple inorganic systems. *All* substrate reductions involve the transfer of two electrons or

multiples thereof (i.e., 4,6,8 . . .). Multielectron substrate reductions may involve the stepwise execution by the enzyme of two-electron processes. Further, about as many protons as electrons are usually transferred to the substrate. One way of viewing the nitrogenase active site is that it can add the elementary particles (H^+ and e^-) of H_2 to the substrate. This may have mechanistic implications.[297]

It is potentially fruitful to pursue the intimate connection between H_2 and the N_2 binding site in nitrogenase. It has been shown unequivocally[298,299] that one H_2 is evolved for each N_2 "fixed" even at 50 atm of N_2, a pressure of N_2 well above full saturation. Moreover, H_2 is a potent inhibitor of N_2 fixation, and under D_2, HD is formed, but only in the presence of N_2. These complex relationships between N_2 and $H_2(D_2)$ have elicited a variety of interpretations.[255,300-302]

Recently, it has been demonstrated that the FeMo protein alone acts as an uptake hydrogenase.[303] Dihydrogen in the presence of [FeMo] causes the reduction of oxidizing dyes such as methylene blue or dichlorophenolindophenol *in the absence of Fe protein*. This is the only known catalytic reaction displayed by the FeMo protein alone. The hydrogen evolution and uptake by [FeMo] suggest that understanding hydrogen interaction with transition-metal/sulfur centers may be crucial to understanding the mechanism of nitrogenase action.

Table 7.8 - Table 7.8 Nitrogenase substrate reactions.[296,374-376]

Two-electron Reductions	
$$2e^{-} + 2H^{+} \rightarrow H_{2}	
$$C_2H_2 + 2e^- + 2H^+ \rightarrow C_2H_4$$	(7.8.1)
$$N_3^- + 2e^- + 3H^+ \rightarrow NH_3 + N_2$$	(7.8.2)
$$N_2O + 2e^- + 2H^+ \rightarrow H_2O + N_2$$	(7.8.3)
	
Four-electron Reductions	
$$HCN + 4e^- + 4H^+ \rightarrow CH_3NH_2$$	(7.8.4)
$$RNC + 4e^- + 4H^+ \rightarrow RNHCH_3$$	(7.8.5)
Six-electron Reductions	
$$N_2 + 6e^- + 6H^+ \rightarrow 2NH_3$$	(7.8.6)
$$HCN + 6e^- + 6H^+ \rightarrow CH_4 + NH_3$$	(7.8.7)
$$HN_3 + 6e^- + 6H^+ \rightarrow NH_3 + N_2H_4$$	(7.8.8)
$$RNC + 6e^- + 6H^+ \rightarrow RNH_2 + CH_4$$	(7.8.9)
$$RCN + 6e^- + 6H^+ \rightarrow RCH_3 + NH_3$$	(7.8.10)
$$NCNH_2 + 6e^- + 6H^+ \rightarrow CH_3NH_2 + 2NH_3$$	(7.8.11)
$$NO_2^- + 6e^- + 6H^+ \rightarrow NH_3$$	(7.8.12)
Multielectron Reductions	

$$RNC \rightarrow (C_2H_6, C_3H_6, C_3H_8) + RNH_2 \tag{7.8.13}$$

$$NCNH_2 + 8e^- + 8H^+ \rightarrow CH_4 + 2NH_3 \tag{7.8.14}$$

11. The Role of ATP

ATP hydrolysis appears to be mandatory, and occurs during electron transfer from [Fe] to [FeMo]. Dissociation of [Fe] and [FeMo] following electron transfer is probably the rate-limiting step in the overall turnover of the enzyme.[255] The fact that reductant and substrate levels do not affect turnover rates is consistent with this finding.

The role of ATP on a molecular level remains one of the great mysteries of the mechanism of nitrogen fixation. As discussed above, the overall thermodynamics of N_2 reduction to NH_3 by H_2 or by its redox surrogate flavodoxin or ferredoxin is favorable. The requirement for ATP hydrolysis must therefore arise from a kinetic necessity. This requirement is fundamentally different from the need for ATP in other biosynthetic or active transport processes, wherein the free energy of hydrolysis of ATP is needed to overcome a thermodynamic limitation.

What is the basis for the kinetic requirement of ATP hydrolysis in nitrogen fixation? To answer this question, we again look at the potential reduction products of the N_2 molecule. Of these, only N_2H_2 (diimide, three potential isomers), N_2H_4 (hydrazine and its mono and dications), and NH_3 (and its protonated form, NH_4^+) are isolable products. (In the gas phase, other species such as N_2H, N_2H_3, or NH_2 also have a "stable" existence.) In the presence of H_2, only the formation of ammonia is thermodynamically favored (Figure 7.26). Clearly, the formation of the intermediate species in the free state cannot occur to any reasonable extent. However, this does not mean that nitrogenase must form NH_3 directly without the formation of intermediates. It is possible for these reactive intermediates to be significantly stabilized by binding to a metal-sulfur center or centers.

Detailed kinetic studies[255,304] have suggested a scheme in which intermediates with bound and probably reduced nitrogen are likely to be present. Rapid quenching experiments in acid solution lead to the detection of hydrazine during nitrogenase turnover.[305] Likewise, studies of inhibition of N_2 fixation by H_2 and the formation of HD under D_2 have been interpreted in terms of a bound diimide intermediate.[306,307] Although a bound "dinitrogen hydride" is likely to be present, its detailed structure remains unknown.