# 7.9: Multisite Redox Enzymes (Part 5)

## The Alternative Nitrogenases

### 1. Vanadium Nitrogenase

The "essentiality" of molybdenum for nitrogen fixation was first reported by Bortels in 1930.[308] This finding led ultimately to the characterization of the molybdenum nitrogenases discussed in the preceding section. Bortels' work has been cited many times, and is often referred to without citation. Following this seminal work, many other Mo-containing enzymes were subsequently sought and found.[25,309] At present more than a dozen distinct Mo enzymes are known, and new ones are continually being discovered.

In addition to the classic 1930 paper, Bortels[310] reported in 1935 that vanadium stimulated nitrogen fixation. In contrast to the 1930 paper, the 1935 paper languished in obscurity. Then, starting in the 1970s, attempts were made to isolate a vanadium nitrogenase. In 1971, two groups reported isolating a vanadium-containing nitrogenase from *A. vinelandii*.[311,312] The interesting notion at this time was that V might substitute for Mo in nitrogenase, *not* that there was a separate system. The isolated enzyme was reported to be similar to the Mo enzyme, but had a lower activity and an altered substrate specificity. One of the groups carefully reinvestigated their preparation, and found small amounts of molybdenum, which were presumed to be sufficient to account for the low activity, although the altered selectivity was not addressed.[313] The vanadium was suggested to play a stabilizing role for [FeMo], allowing the small amount of active Mo-containing protein to be effectively isolated. Apparently the possibility was not considered that a truly alternative nitrogenase system existed, whose protein and metal centers both differed from that of the Mo nitrogenase.

The unique essentiality of molybdenum for nitrogenase fixation went unchallenged until 1980, when it was demonstrated[314] that an alternative nitrogenfixation system could be observed in *A. vinelandii* when this organism was starved for molybdenum.[315] Despite skepticism from the nitrogenase research community, it was eventually shown that even in a mutant from which the structural genes for the Mo nitrogenase proteins (*nif* H, D, and K) had been deleted, the alternative system was elicited upon Mo starvation. In 1986, two groups[316-321] isolated the alternative nitrogenase component proteins from different species of *Azotobacter*, and demonstrated unequivocally that one component contained vanadium and that neither component contained molybdenum.

One of the two components of the V-nitrogenase system is extremely similar to the Fe protein of nitrogenase. This similarity is evident in the isolated proteins from *A. vinelandii*[316] and in the genetic homology between *nif* H (the gene coding for the subunit of the Fe protein in the Mo-nitrogenase system) and *nif* H* (the corresponding gene in the V-based system). Both Fe proteins have an $\alpha_2$ subunit structure, and contain a single $Fe_4S_4$ cluster that is EPR-active in its reduced state.

The FeV proteins from *Azotobacter vinelandii* and *Azotobacter chroococcum* each have an $\alpha_2\beta_2\delta_2$ subunit structure.[322] Metal composition and spectroscopic comparisons between the FeMo and FeV proteins are shown in Table 7.9. Although there is the major difference involving the presence of V instead of Mo in the FeV protein and in the probable presence of the small $\delta$ subunits (13 kDa), the two nitrogenase systems are otherwise quite similar.[322] In each, a system of two highly oxygen-sensitive proteins carries out an ATP-dependent $N_2$ reduction with concomitant $H_2$ evolution. The Fe proteins have the same subunit structure and cluster content, and are spectroscopically very similar. The V versions of the larger protein have somewhat lower molecular weights than their Mo analogues, and by MCD spectroscopy seem to contain P-like clusters.[318] The FeV site still may be an $S = \frac{3}{2}$ center (by EPR, although its EPR differs significantly from that of the FeMo center).[323] The V-S and V-Fe distances as measured by EXAFS[324,325] are similar to those in thiocubane $VFe_3S_4$ clusters and to Mo-S and Mo-Fe distances like those in [FeMo], which are in turn similar to those in $MoFe_3S_4$ thiocubanes. Likewise, XANES[324,325] indicates $VS_3O_3$ type coordination in [FeV] nitrogenase similar to the $MoS_3O_3$ coordination suggested by XANES for FeMoco. The "FeV cofactor" is extractable into NMF, and can reconstitute the *nif* $B^-$, FeMoco-deficient mutant of the Mo system.[326] Despite the substitution of V for Mo, the proteins and their respective M-Fe-S sites do not differ drastically. However, the compositional changes do correlate with altered substrate reactivity.

A major difference between the V and Mo enzymes lies in substrate specificity and product formation.[321] As is clearly shown in Table 7.9, the FeV nitrogenase has a much lower reactivity toward acetylene than does the Mo system. Furthermore, whereas the FeMo system exclusively produces ethylene from acetylene, the FeV system yields significant amounts of the four-electron reduction product, ethane.[321] The detection of ethane in the acetylene assay may prove a powerful technique for detecting the presence of the V nitrogenase in natural systems.[322] Moreover, this reactivity pattern is found in the *nif* $B^-$ mutant reconstituted with FeVco, indicating that the pattern is characteristic of the cofactor and not the protein.[326] The reactivity change upon going from Mo to V in otherwise similar protein systems clearly adds weight to the implication of the M-Fe-S center (M = V or Mo) in substrate reduction.

Table 7.9 - Comparison of alternative nitrogenase proteins[a]

a) Av1 is the FeMo protein of *Azotobacter vinelandii*, Av1* is the FeV protein of *A. vinelandii* and Ac1* is the FeV protein of *A. chroococcum*. Data from References 317,377, and 319, respectively.

b) Atoms per molecule.

c) nmol product/min/mg of protein.

| Property | Av1[47] | Av1*[47] | Ac1*[50] |
|---|---|---|---|
| Molecular Weight | 240,000 | 200,000 | 210,000 |
| Molybdenum[b] | 2 | < 0.05 | < 0.06 |
| Vanadium[b] | — | 0.7 | 2 |
| Iron[b] | 30-32 | 9.3 | 23 |
| Activity[c] | | | |
| $H^+$ | 2200 | 1400 | 1350 |
| $C_2H_2$ | 2000 | 220 | 608 |
| $N_2$ | 520 | 330 | 350 |
| EPR g values | 4.3 | 5.31 | 5.6 |
| | 3.7 | 4.34 | 4.35 |
| | 2.01 | 2.04 | 3.77 |
| | | 1.93 | 1.93 |

## 2. The All-iron Nitrogenase[322]

The first sign that there is yet another alternative nitrogenase again came from genetic studies. A mutant of *A. vinelandii* was constructed with deletions in both *nif* HDK and *nif* H*D*K*, i.e., the structural genes for the Mo and V nitrogenases, respectively. Despite lacking the ability to make the two known nitrogenases, the mutant strain nevertheless was able to fix nitrogen, albeit poorly. Moreover, this mutant strain's nitrogenase activity was clearly inhibited when either Mo or V was present in the culture medium. Preliminary studies indicate that the nitrogenase proteins produced by this organism are closely related to those previously isolated. A 4Fe-4S Fe-protein *nif* $H^\dagger$ and a protein due to *nif* $D^\dagger$ was produced. The latter appeared to contain no stoichiometric metal other than iron. Symmetry of nomenclature would suggest calling this the FeFe protein and its cofactor FeFeco. Interestingly, this nitrogenase seems to be the poorest of the set in reducing $N_2$ and makes ethane from ethylene. The finding of the all-iron nitrogenase, if fully confirmed, will add significantly to the comparative biochemistry of nitrogen fixation. Speculatively, one might suggest that the concomitant absence of V and Mo suggests that nitrogen fixation need not directly involve the noniron heterometal in the cofactor cluster. This result may explain the lack of direct implication of Mo in the nitrogen fixation mechanism, despite many years of intense effort by workers in the field. (The above discussion should be taken *cum grana salis* until the existence of the all-iron nitrogenase is confirmed.)

## 3. Model Systems

Three types of model systems for nitrogenase may be considered. First, there are transition-metal sulfide clusters that resemble the FeMoco or FeVco centers of the active proteins. Although there has been significant progress, there are not yet any definitive models (as there are for $Fe_2S_2$ and $Fe_4S_4$). A second approach uses the reactions of $N_2$ and related substrates or intermediates with metal centers in order to gain insights into the way in which transition-metal systems bind $N_2$ and activate it toward reduction. Here to date the most reactive systems bear little direct chemical resemblance to the nitrogenase active sites. Nevertheless, these systems carry out *bona fide* nitrogen fixation from which one may learn the various ways in which $N_2$ can be activated. Finally, there are other inorganic systems that display some of the structural and possibly some of the reactivity characteristics of the nitrogenase active sites without binding or reducing $N_2$ or precisely mimicking the active center. We may nevertheless be able to learn effectively about nitrogenase reactivity from these interesting chemical systems.

### a. Transition-metal Sulfide Models for Nitrogenase Sites

Although there has been great activity in synthetic Fe-S cluster chemistry, there is to date no example of a spectroscopic model for the P-cluster sites in nitrogenase. If the P-clusters are indeed asymmetrically bound high-spin $Fe_4S_4$ clusters, then the recent work on high-spin versions of $Fe_4S_4$ clusters[327] and site-selectively derivatized $Fe_4S_4$ centers[143] may hint that appropriate model systems are forthcoming.

### b. Fe-M-S Cluster Models for FeMoco

Despite the importance of P-clusters, the modeling of the FeMoco center has properly received the most attention. The significant structural parameters that any model must duplicate are the Mo-S and Mo-Fe distances detennined by EXAFS. Spectroscopically, the S = $\frac{3}{2}$ EPR signal provides a stringent feature that model systems should aspire to mimic.

Many FeMoS clusters have been prepared in the quest to duplicate the FeMoco center, but *none* of the chemically synthesized clusters can reactivate the (UW-45 or *Nif* $B^-$) cofactor-less mutants, perhaps because of their lack of homocitrate, which only recently has been discovered as a key component of FeMoco. Undoubtedly, new FeMoS clusters containing homocitrate will be prepared, and perhaps these will activate the mutant proteins, thereby revealing a close or full identity with FeMoco.

Despite the absence of homocitrate, some interesting model systems have been investigated. It is beyond the scope of this chapter to give a comprehensive account of FeMoS chemistry. We concentrate on the so-called "thiocubane" model systems. Heterothiocubane models were first synthesized using self-assembly approaches analogous to those used for the simpler Fe-S model systems. The reaction[328-330a]

$$MoS_4^{2-} + Fe^{3-} + SR^- \rightarrow (MoFe_3S_4)_2(SR)_9^{3-} \; and \; (MoFe_3S_4)_2Fe(SR)_{12}^{3-,4-} \tag{7.17}$$

uses tetrathiomolybdate, $MoS_4^{2-}$,as the source of Mo, and leads to the double cubane structures shown in Figure 7.32A,B. The $Fe_7Mo_2S_8$ structure proved particularly interesting, since it was possible to complex the central ferric iron atom with substituted catecholate ligands[331,332] and eventually isolate a single thiocubane unit (Figure 7.32C). Significantly, the single unit has S = $\frac{3}{2}$ and Mo-S and Mo-Fe distances that match precisely those found by EXAFS for the M center of nitrogenase. Single cubes with $VMo_3S_4$ cores have also been prepared.[160,160a] Although the single thiocubanes display spectroscopic similarity and distance identity with FeMoco, they are not complete models. They are stoichiometrically Fe and S deficient, lack homocitrate, and most importantly, fail to activate the UW-45 and *Nif* $B^-$ mutants.

Other interesting FeMoS (and FeWS) clusters with structurally distinct properties are shown in Figure 7.33. These include the "linear" $(MoS_4)_2Fe^{3-}$ ion, the linear $(WS_4)_2Fe[HCON(CH_3)_2]_2^{2-}$ ion, the linear $Cl_2FeS_2MS_2Fe_2CI_2^{2-}$ (M = Mo, W), the "linear" $(MoS_4)_2Fe_2S_2^{4-}$ ion, the trigonal $(WS_4)_3Fe_3S_2^{4-}$, the capped thioprismane $Fe_6S_6X_6[M(CO)_3]_2^{3-}$ (X = CI, Br, I; M = Mo, W), and the organometallic clusters $MoFe_6S_6(CO)_{16}^{2-}$, $MoFe_3S_6(CO)_6(PEt_3)3$, and $MoFe_3S_6(CO)_6^{2-}$. Structures suggested for FeMoco based on these and other chemically synthesized transition metal sulfides and on spectroscopic studies of the enzyme are shown in Figure 7.31.

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