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VTVH-MCD Study of the Δ*nifB*Δ*nifZ* MoFe Protein from *Azotobacter vinelandii*

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Nitrogenase catalyzes the key step in the global nitrogen cycle: the reduction of atmospheric dinitrogen to bioavailable ammonia. The Mo-nitrogenase of *Azotobacter vinelandii* is composed of two proteins: the Fe protein, an α_2-dimer bridged by a [4Fe-4S] cluster, and the MoFe protein, an $\alpha_2\beta_2$-tetramer, containing, per $\alpha\beta$-dimer, a P-cluster ([8Fe-7S]) at the α/β-subunit interface and a FeMoco ([Mo-7Fe-9S-X], where X = C, O, or N) within the α-subunit. The Fe protein (encoded by *nifH*) not only serves as an obligate electron donor for the MoFe protein during catalysis but also functions as an important maturation factor in the biosynthesis of the MoFe protein.[1]

Assembly of the MoFe protein is a well-coordinated process involving multiple events, including (1) the biosynthesis of α- and β-polypeptides of the MoFe protein; (2) the maturation of P-cluster at its target location within the MoFe protein; and (3) the incorporation of FeMoco (synthesized elsewhere) into its binding site in the MoFe protein. This complicated process requires the participation of a number of *nif* genes, and systematic deletions of these *nif* genes have led to the identification of several intermediates at different stages of the biosynthesis of the MoFe protein.[1] One of them, generated by *nifB* deletion, does not contain FeMoco;[2] however, the crystal structure of this protein shows that it contains intact P-clusters[3] at the same concentration as that of the holo MoFe protein. In contrast, another FeMoco-deficient form of the MoFe protein, generated by *nifH* deletion, contains different P-cluster species. EXAFS[4] and VTVH-MCD[5] spectroscopic studies suggest the presence of paired [4Fe-4S]-like clusters in this protein, which are presumably precursors to the [8Fe-7S] P-cluster. Finally, a third form of the FeMoco-deplete MoFe protein has been obtained through simultaneous deletions of *nifB* and *nifZ* genes. Such a form of the MoFe protein is, yet again, different from those derived from *nifB* or *nifH* deletion, as EPR analysis indicates the coexistence of both [4Fe-4S]-type clusters and P-clusters in this protein. Moreover, combined activity and metal analyses suggest this protein contains approximately half of the P-cluster content of the Δ*nifB* MoFe protein. Taken together, these results point to a plausible biosynthetic flow of the MoFe protein from the intermediate represented by the *nifH*-deletion MoFe protein (which only contains precursors to P-clusters) to that represented by *nifB*/*nifZ*-double deletions (which contains a mixture of precursors and P-clusters) and, eventually, to that represented by the *nifB*-deletion MoFe protein (which only contains fully assembled P-clusters). Further, the presence of mixed P-cluster species (presumably at different maturation levels) in the *nifB*/*nifZ*-deletion MoFe protein suggests an interesting, "unsynchronized" assembly route undertaken by the P-clusters of the MoFe protein.

Here we present an MCD spectral investigation of the electronic structures of the metal clusters at the P-cluster sites of the *nifB*/*nifZ*-deletion MoFe protein. Three His-tagged MoFe proteins from *A. vinelandii* strains were used in this study: (1) an *nifB*/*nifZ*-deletion MoFe protein (designated Δ*nifB*Δ*nifZ* MoFe protein) from strain YM6A; (2) an *nifB*-deletion MoFe protein (designated Δ*nifB* MoFe protein) from strain DJ1143; and (3) an *nifH*-deletion MoFe protein (designated Δ*nifH* MoFe protein) from strain DJ1165. The absence of FeMoco from these proteins greatly simplifies the interpretation of MCD spectra, allowing unambiguous assessments of signals arising solely from the P-cluster sites. MCD spectra were measured as previously described.[5] Each protein sample contained 55% (v/v) glycerol as a glassing agent for low-temperature MCD studies. Spectra were expressed as the difference between the molar extinction coefficients, or $\Delta\varepsilon$, of right and left circularly polarized lights and normalized per unit in $M^{-1}cm^{-1}$. Magnetization curves were plotted as the percentage of maximum magnetization (i.e., percentage of MCD intensity relative to saturation) versus $\beta B/2kT$, where β is the Bohr magneton and B is the magnetic flux.

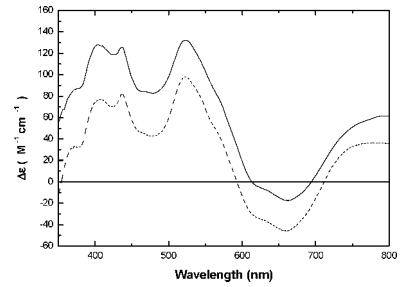

Figure 1. Comparison of MCD spectra of dithionite-reduced Δ*nifB*Δ*nifZ* (solid line) and Δ*nifH* (dashed line) MoFe proteins. Spectra were recorded at a temperature of 1.5 K and a magnetic field 6 T.

The MCD spectrum of the dithionite-reduced Δ*nifB*Δ*nifZ* MoFe protein (Figure 1, solid line) reveals positive bands at 400, 440, 520, and 800 nm (with the band at 800 nm relatively broad in shape) and a negative band at 670 nm. These inflections are typical of [4Fe-4S] cluster(s) in the 1+ oxidation state. Further, an increase of signal intensity can be observed upon a decrease in temperature (data not shown), signifying ground-state paramagnetism of the active species.

Interestingly, nearly identical inflections are observed in the MCD spectrum of the dithionite-reduced Δ*nifH* MoFe protein (Figure 1, dashed line), suggesting a similarity between the respective P-cluster species in Δ*nifH* and Δ*nifB*Δ*nifZ* MoFe proteins. Previous EXAFS analysis of the Δ*nifH* MoFe protein[4] predicts a pair of [4Fe-4S]-like clusters acting as a P-cluster precursor of the mature [8Fe-7S] structure. MCD spectral analysis of the same protein[5] is consistent with this interpretation and, additionally, shows the [4Fe-4S] pair to be spin coupled (i.e., close neighbors) in different oxidation states (1+ and 0). As such, the Δ*nifB*Δ*nifZ* MoFe protein could also contain a latent P-cluster (i.e., precursor) that is identical in

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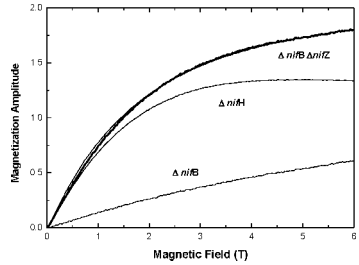

Figure 2. Molar magnetization curves of Δ*nifB*, Δ*nifH*, and Δ*nifB*Δ*nifZ* MoFe proteins. Simulation of the magnetization curve of the Δ*nifB*Δ*nifZ* MoFe protein (thick line) predicts approximately equal contributions from the curves of the Δ*nifB* and Δ*nifH* MoFe proteins.

electronic structure to that found in the Δ*nifH* MoFe protein. Consistent with this argument, the EPR spectrum of the Δ*nifB*Δ*nifZ* MoFe protein[6] shows an $S = {}^1/_2$ signal with *g*-factors that are identical to those of the Δ*nifH* MoFe protein, further suggesting the presence of the same type of [4Fe-4S] clusters in both proteins.

Despite their similarities, the spectrum of the Δ*nifB*Δ*nifZ* MoFe protein is positively shifted compared to that of the Δ*nifH* MoFe protein, likely due to the presence of an underlying, broad, positive signal in the spectrum of the former (Figure 1). This observation indicates that the P-cluster composition of the Δ*nifB*Δ*nifZ* MoFe protein is different than that of the Δ*nifH* MoFe protein. Indeed, the EPR spectrum of the oxidized Δ*nifB*Δ*nifZ* MoFe protein[6] shows a characteristic signal of the P-cluster, which is present in the spectrum of the oxidized Δ*nifB* MoFe protein but absent from that of the oxidized Δ*nifH* MoFe protein.[7] Combined results from these studies suggest that the Δ*nifB*Δ*nifZ* MoFe protein contains two types of clusters: (1) a native (8Fe) P-cluster identical to that in the Δ*nifB* MoFe protein and (2) a latent (2 × 4Fe) P-cluster electronically identical to that in the Δ*nifH* MoFe protein. If this cluster assignment is correct, we should be able to simulate the spectral parameters of the Δ*nifB*Δ*nifZ* MoFe protein using the parameters of the Δ*nifB* and Δ*nifH* MoFe proteins.

The MCD spectrum of the Δ*nifB* MoFe protein has no distinct features or inflections.[5] Therefore, using it along with the spectrum of the Δ*nifH* MoFe protein to simulate the spectrum of the Δ*nifB*Δ*nifZ* MoFe protein would not be definitive. The magnetization curves of these proteins, on the other hand, are more informative. As shown in Figure 2, the magnetization curve of the dithionite-reduced Δ*nifB* MoFe protein is typical of a diamagnetic system being relatively linear with the magnetic field. In contrast, the magnetization curve of the Δ*nifH* MoFe protein plateaus when the magnetic field reaches a certain strength. This curve can be well simulated by a pure $S = {}^1/_2$ spin system,[5] which is further consistent with the presence of only one $S = {}^1/_2$ signal in the EPR spectrum of the Δ*nifH* MoFe protein.[7] The magnetization curve of the Δ*nifB*Δ*nifZ* MoFe protein is more complex. While it resembles more closely the shape of the magnetization curve of the Δ*nifH* MoFe protein, it never reaches saturation and, in this way, mimics the behavior of the magnitization curve of the Δ*nifB* MoFe protein. These observations imply that the Δ*nifB*Δ*nifZ* MoFe protein may

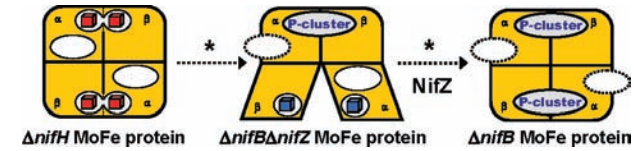

Figure 3. Model of the stepwise assembly of P-clusters in MoFe protein. Circles represent the FeMoco binding sites, which can be converted from "closed" (solid circles) to "open" (dashed circle) conformation upon P-cluster formation. Cubes represent P-cluster precursors that are consisted of paired [4Fe-4S] clusters. Steps requiring Fe protein and MgATP are indicated by asterisks.

contain cluster types like those in both Δ*nifB* and Δ*nifH* MoFe proteins. Using a nonlinear regression, the molar magnetization curve of the Δ*nifB*Δ*nifZ* MoFe protein can be easily simulated (Figure 2, thick line) by factored contributions from the magnetization curves of the Δ*nifB* MoFe protein and the Δ*nifH* MoFe protein in an approximate ratio of 1:1 (actual ratio of 0.97:0.89). This result strengthens the hypothesis previously implied by the EPR spectral and enzymatic analyses that the Δ*nifB*Δ*nifZ* MoFe protein is a "half assembled" form, in which one $\alpha\beta$-dimer contains a fully assembled P-cluster and the other, a P-cluster precursor. Further, it supplies the first piece of evidence that the P-cluster precursor in the Δ*nifB*Δ*nifZ* MoFe protein is electronically identical to those identified earlier in the Δ*nifH* MoFe protein.

The combined outcome of the current MCD study and previous investigations of the biosynthetic intermediates of the MoFe protein suggests a stepwise assembly mechanism of this protein that involves the sequential formation of P-clusters, one at a time, in the two $\alpha\beta$-halves of the protein (Figure 3). Fusion of the "first" pair of [4Fe-4S] clusters into a mature P-cluster likely forces the "second" pair of [4Fe-4S] clusters into an unfavorable conformation, and NifZ may be involved at this stage to reposition the clusters in the correct orientation for the subsequent coupling. Formation of both P-clusters requires the Fe protein and MgATP, which concomitantly "opens" up the FeMoco binding site, likely through the Fe protein-induced conformational changes. Once the P-clusters are formed, FeMoco can be inserted to complete the assembly process of the MoFe protein. How NifZ functions in this process and why the MoFe protein has to be synthesized one-half at a time is unknown but is the focus of further investigation.

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