Construction and Characterization of Hybrid Component 1 from V-Nitrogenase Containing FeMo Cofactor

Virginia G. Moore, Roland C. Tittsworth, and Brian J. Hales*

Department of Chemistry
Louisiana State University
Baton Rouge, Louisiana 70803-1804

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It is now well established that the enzyme responsible for nitrogen fixation can exist in three different forms, a Mo and Fe form called Mo-nitrogenase, a V and Fe form called V-nitrogenase,[1,2] and a third form, nitrogenase-3, which may only contain Fe.[3] All three enzymes consist of two separable proteins, called components 1 and 2, with component 2 acting as the obligate electron donor to component 1, where substrate reduction occurs. While little is currently known about nitrogenase-3, the component 1 proteins of Mo- and V-nitrogenase have been shown to be very similar. For example, both proteins possess cofactor clusters (FeMoco and FeVco, respectively)[4,5] which are presumed to function as the sites of substrate reduction. MCD[6] and X-ray absorption[7−11] spectroscopic studies of these two cofactors have revealed only minor differences. Furthermore, the amino acid sequences of the major polypeptide subunits from both component 1 proteins are highly homologous, especially in the vicinity of their respective cofactor clusters.[12] It is thus surprising that the enzymology (e.g., variations in electron allocation and reductive products) of these two forms of nitrogenase differs significantly.[13−16] Obviously subtle variations in the structure of these proteins or their cofactors have dramatic consequences on their enzymology. We have investigated the structural basis of the mechanistic differences between Mo- and V-nitrogenase through the construction, purification, and characterization of a hybrid component 1 protein (called Av1′(FeMoco)) which possesses the polypeptides of the VFe protein (Av1′) from *Azotobacter vinelandii* and the cofactor clusters (FeMoco) of the MoFe protein.

The FeMoco clusters were extracted from purified MoFe protein (Av1) from *A. vinelandii* using the procedure of Orme-Johnson and co-workers.[17] The cofactorless form of the VFe protein from *A. vinelandii* was obtained from strain LS25 (kindly constructed by Prof. Paul Bishop, North Carolina State University), which contains a deletion in *nifHDK* (the genes encoding the structural polypeptides of Mo-nitrogenase), a resistance mutation to diazotropic growth in the presence of tungsten, and a Tn5 transposon inserted into *nifB* (a gene necessary for cofactor construction). Cells of LS25 were grown on Burk's medium supplemented with 20 μM V_2O_5 and 5 mM NH_4Cl and harvested 3 h following depletion of the ammonia, as determined by colorimetric assay of the medium using Nessler's reagent. Crude extract from these cells exhibited a normal component 2 specific activity of 55 nmol of C_2H_2 reduced min^{-1} mg^{-1} of protein and no detectable component 1 activity.

To construct Av1′(FeMoco), 150 mL of concentrated crude extract of derepressed LS25 cells was mixed with an ATP regenerating mixture (60 mL) consisting of 1.7 mM ATP, 3.3 mM $MgCl_2$, 20 mM phosphocreatine, and 0.0084 mg/mL creatine phosphokinase in 25 mM TES buffer containing 2 mM dithionite. Extracted FeMoco in dimethylformamide (specific activity when reconstituted into apo-Av1 = 220 nmol of C_2H_4 produced min^{-1} $nmol^{-1}$ of Mo) was added (34 μL/mL extract) along with purified component 2 (Av2) to aid in cofactor insertion. Because component 2 preparations often contain contaminating component 1, the Av2 used for cofactor insertion was purified from derepressed UW45 cells (wild-type *A. vinelandii* cells with *nifB*$^-$ mutation) possessing no component 1 activity. Following a 20 min incubation with shaking at 30 °C, Av1′(FeMoco) was purified from the reconstituted mixture using the same procedure[2] developed to purify Av1′ with the elimination of the 52 °C heat step. Metal analysis using ICP emission spectroscopy showed a Mo:Fe ratio of 1:15 ± 2, as expected for an intact protein containing 2 P clusters and 2 FeMoco. No V was detected in these preparations. SDS PAGE of Av1′(FeMoco) revealed predominantly (>90%) the polypeptide bands of Av1′ with no detectable bands assignable to Av1. Final specific activity was 550 nmol of H_2 produced min^{-1} mg^{-1} of protein.

Figure 1 shows the low-temperature EPR spectra of purified Av1, Av1′(FeMoco), and Av1′. The single $S = {}^3/_2$ species (g = 4.32, 3.68, and 2.01) in the spectrum of Av1 arises from protein-bound FeMoco while the analogous $S = {}^3/_2$ species (g = 5.68 and 5.45) in the Av1′ spectrum has been assigned to FeVco. The $S = {}^3/_2$ signal (g = 4.65 and 3.49; 0.9 spins per Mo) in the spectrum of Av1′(FeMoco) presumably arises from FeMoco in the Av1′ protein. During enzymatic reduction, the amplitude of this signal in Av1′(FeMoco) decreased by about 50%, similar to the decrease of the $S = {}^3/_2$ signals observed during turnover with Av1 or Av1′ and consistent with the $S = {}^3/_2$ signal in Av1′(FeMoco) being assigned to FeMoco. The rhombicity of this signal in Av1′(FeMoco) ($|E|/|D|$ = 0.093) is intermediate between that of Av1 ($|E|/|D|$ = 0.053) and Av1′ ($|E|/|D|$ = 0.29) and suggests that the rhombicity of the protein-bound cofactor is a property of both the cofactor and the protein. Furthermore, the line shape of the $S = {}^3/_2$ signal in Av1′-(FeMoco) is broader than in either Av1 or Av1′. This increase in broadness may arise from an overlap of the spectra of slightly different cofactor signals, suggesting that FeMoco may not have as well-defined an orientation in Av1′(FeMoco) as it does in

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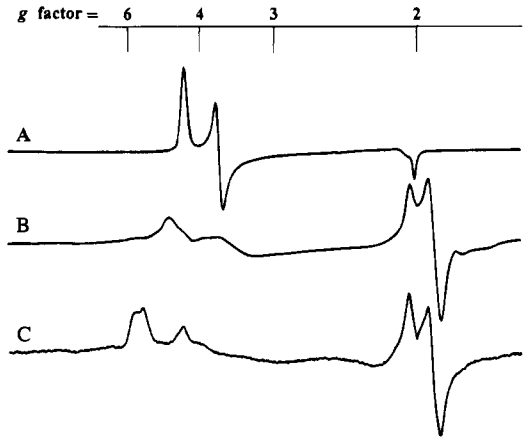

Figure 1. Low-temperature EPR spectra of Av1 (A), Av1′(FeMoco) (B), and Av1′ (C) as isolated in the presence of 2.0 mM dithionite. Spectral pararneters: (A) modulation amplitude, 12.6 G; microwave power, 20 mW; temperature, 3.3 K; gain, 6.3 × 10^4; protein concentration, 37 mg/mL; (B) modulation amplitude, 10 G; microwave power, 20 mW; temperature, 3.3 K; gain, 1 × 10^5; protein concentration, 57 mg/mL; (C) modulation amplitude, 10 G; microwave power, 20 mW; temperature 7 K; gain, 2.5 × 10^5; protein concentration, 41 mg/mL.

Av1. This suggestion is interesting since the Av1′ α polypeptide, which binds the cofactor, contains[12] many of the same amino acids present in the α subunit of Av1 (i.e., Gln191, His195, Cys275, Ser278, Gly356, Gly357, Arg359, and His442), which have been associated with cofactor ligation and interaction.[18,19] Therefore, since these amino acids are conserved in both enzyme systems, other amino acids which are unique to each enzyme may be used to fine-tune the size of the protein pocket for the different cofactors. The EPR spectrum of Av1′-(FeMoco) also exhibits an axial $S = {}^1/_2$ signal in the $g = 2$ region. This signal is present in the spectrum of Av1′ but not in the Av1 spectrum, suggesting that it may originate in the [Fe_8–S_{7-8}] P clusters of Av1′. However, recent Mössbauer studies[20] on Av1′ show that the P clusters are diamagnetic, thus implying that the $S = {}^1/_2$ signal originates in the cofactor. Therefore, the origin of the $g = 2$ signal is still unknown.

Using saturating concentrations of nitrogenase component 2, the enzymologies of Av1, Av1′, and Av1′(FeMoco) were investigated (Table 1), revealing several surprising results. First of all, purified Av1′(FeMoco) fixes nitrogen with an electron allocation similar to that of Av1. (This result can be compared to the enzymology of the hybrid Kp1(FeVco) constructed by inserting FeVco into the cofactorless MoFe protein (Kp1) from *Klebsiella pneumoniae* which yielded a protein unable to fix nitrogen.[21]) In contrast, the electron allocation for the reduction of acetylene by Av1′(FeMoco) mimics the pattern of Av1′ and not that of the MoFe protein. Furthermore, Av1′(FeMoco) reduces acetylene to *both* ethylene and ethane, a phenotype associated with V-nitrogenase.[13,14] In all of these experiments, the electron allocation stated is the percent relative to that of H_2 evolution in the absence of an added substrate.

Table 1. Percent of Electrons as Product at 30 °C

substrate	product	Av1	Av1′	Av1′(FeMoco)
N_2[a]	NH_3[b]	72	50	70
	H_2[c]	28	50	30
C_2H_2[d]	C_2H_4[e]	95	31	35
	H_2	5	66	55
	C_2H_6[f]	0	3	10
K_i(CO) in atm		0.0003	0.03	0.03
CO-EPR?[g]		yes	no	no

[a] Gas phase contained 100% N_2. [b] NH_3 measured using a colorimetric determination.[2] [c] H_2 determined using a Gow-Mac gas chromatograph equipped with a thermal conductivity detector and a 5 Å molecular sieve column. [d] Gas phase contained 10% C_2H_2 in Ar. [e] C_2H_4 measured using a Varian (Model 3700) gas chromatograph equipped with a flame ionization detector and a Porapac type T column. [f] C_2H_6 measured using a Varian (Model 3700) gas chromatograph equipped with a flame ionization detector and an activated alumina column. [g] EPR spectroscopy was used to determine whether $S = {}^1/_2$ CO-induced signals were generated during enzymatic turnover conditions.

CO inhibition was also investigated. For both Mo-nitrogenase and V-nitrogenase, CO has the ability to inhibit reduction of all external substrates but has no effect on the reduction of H^+ to H_2. This characteristic is preserved in Av1′(FeMoco). We recently have demonstrated (Cameron and Hales, manuscript in preparation) that CO is a less potent inhibitor of Av1′ compared to Av1 with a K_i approximately 100× larger. It has been demonstrated[22,23] that, during CO inhibition of Mo-nitrogenase, two different $S = {}^1/_2$ EPR signals are generated, one (g = 2.1, 1.98, and 1.92) at low CO concentrations ($0.001 \leq p(CO) \leq 0.05$ atm) and another (g = 2.17, 2.1, and 2.05) at higher CO concentrations. Broadening induced by ^{57}Fe enrichment of component 1 has demonstrated[22] that these signals arise from either the P cluster or the cofactor. No similar signals are generated by V-nitrogenase (Cameron and Hales, manuscript in preparation). Therefore, the generation of CO-induced EPR signals appears to be unique to Mo-nitrogenase. Because of this, it is mechanistically important to determine how CO affects both the enzymology and spectroscopy of Av1′(FeMoco). By measurement of the inhibition of acetylene reduction at various CO concentrations, the K_i for CO inhibition of Av1′(FeMoco) was determined (Table 1) to be identical to that of Av1′. Furthermore, the EPR spectrum of Av1′(FeMoco) under the conditions of enzymatic turnover in the presence of 1.0 atm of CO did not exhibit any CO-induced $S = {}^1/_2$ EPR signals. Therefore, like acetylene reduction, the phenotype for CO inhibition of Av1′(FeMoco) mimics that of the protein and not the metal cofactor. In summary, these studies suggest that the component 1 protein structure influences the enzymology of C_2H_2 and CO. On the other hand, N_2 reduction by Av1′-(FeMoco) mimics the cofactor and not the protein, suggesting that N_2 interacts mainly with the cofactor atoms. The recent suggestion of N_2 binding and reduction within the cofactor cage[24] is one example of this type of interaction.

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