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MOLECULAR INSIGHTS INTO NITROGENASE FeMoco INSERTION – THE ROLE OF His 274 and His 451 OF MoFe PROTEIN α SUBUNIT

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Abstract

The final step of FeMo cofactor (FeMoco) assembly involves the insertion of FeMoco into its binding site in the molybdenum-iron (MoFe) protein of nitrogenase. Here we examine the role of His α 274 and His α 451 of *Azotobacter vinelandii* MoFe protein in this process. Our results from combined metal, activity, EPR, stability and insertion analyses show that mutations of His α 274 and/or His α 451, two of the histidines that belong to a so-called His triad, to small uncharged Ala specifically reduce the accumulation of FeMoco in MoFe protein. This observation indicates that the enrichment of histidines at the His triad is important for FeMoco insertion and that the His triad potentially serves as an intermediate docking point for FeMoco through transitory ligand coordination and/or electrostatic interaction.

1. Introduction

Nitrogenase catalyzes the nucleotide-dependent reduction of the inert atmospheric dinitrogen to the bioavailable form of ammonia [for recent reviews see refs. 1–5]. The molybdenum (Mo) nitrogenase of *Azotobacter vinelandii* is a two-component system, comprising the iron (Fe) protein and the molybdenum iron (MoFe) protein. The Fe protein (also designated Av2) is a homodimer (encoded by *nifH*), with one ATP binding site per subunit and a single [4Fe-4S] cluster bridged between the subunits. The MoFe protein (also designated Av1) is an $\alpha_2\beta_2$ -tetramer (encoded by *nifD* and *nifK*), containing two unique metal clusters per $\alpha\beta$ subunit pair: the [8Fe-7S] P-cluster [6], which is bridged between α - and β -subunits and ligated to six Cys ligands; and the [Mo-7Fe-9S-X-homocitrate]¹ FeMo cofactor (FeMoco)[7], which is located within the α -subunit and bound to the protein by Cys α 275 at the terminal Fe and His α 442 at the opposite Mo that is further coordinated by an endogenous homocitrate. Nitrogenase catalysis involves complex association/dissociation between the Fe protein and the MoFe protein, during which process electrons, concomitant with ATP hydrolysis by the Fe protein, are sequentially transferred from the [4Fe-4S] cluster in the Fe protein through the P-cluster to the FeMoco in the MoFe protein, where substrate reduction takes place.

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¹The identity of X is unknown but it is considered to be C, O or N [7].

The biosynthesis of nitrogenase component proteins and complex metal clusters are controlled by the nitrogen fixation (*nif*) genes [8]. The assembly of MoFe protein occurs in two steps. First, a P-cluster-replete, yet FeMoco-depleted form is synthesized in a process that involves the expression of *nifD* and *nifK*—the structural genes encoding the α - and β -subunits of the MoFe protein, and the formation of P-cluster—likely through fusion of its substructural, [4Fe-4S] cluster units at the target location (*in situ*) in the protein [8–10]. Then, the FeMoco—assembled outside of the MoFe protein (*ex situ*) in a process that involves the participation of *nifB*, *nifE*, *nifN*, *nifH*, *nifV* and *nifQ*, is inserted into its binding site, resulting in the formation of an active, holo form of MoFe protein [8]. The *ex situ* assembly of FeMoco is presumably initiated with the formation of an Fe/S core on NifB, followed by the relocation and rearrangement of the core on the NifEN complex [11–14]. Recently, we identified a Mo-free, NifEN-bound FeMoco precursor that closely resembled the core structure of the mature FeMoco [11,12]. We also showed that, coupled to the hydrolysis of ATP, the Fe protein inserted Mo and homocitrate into the precursor while it was still bound to NifEN, resulting in a fully complemented cluster that could be inserted into the MoFe protein through direct protein-protein interaction [13,14]. These recent developments on *ex situ* FeMoco biogenesis not only clarify certain aspects of the cluster assembly in nitrogenase, they may also provide useful insights into the biosynthetic strategy of complex metal clusters in other systems [15–17].

On the other hand, the mechanism of the final step of FeMoco assembly—namely, how FeMoco is inserted into its target binding site in the MoFe protein, remains largely unknown. Much of the current understanding in this regard comes from the crystallographic studies of a Δ *nifB* MoFe protein from a *nifB*-deletion strain of *A. vinelandii* [18]. In agreement with the genetically-based theory that *nifB* is the starting point of FeMoco biosynthesis, the Δ *nifB* MoFe protein is FeMoco-deficient, yet it contains normal P-clusters [18]. More importantly, the Δ *nifB* MoFe protein can be readily reconstituted, *in vitro*, into an active holo protein by addition of isolated FeMoco, suggesting that it represents a physiologically relevant snapshot of MoFe protein right before the final insertion of FeMoco [18]. In contrast to the wild-type MoFe protein, the Δ *nifB* MoFe protein contains a positively charged funnel that could allow the entry and navigation of the negatively charged FeMoco toward its target location [18]. Such a FeMoco insertion funnel is created as a result of major structural rearrangement of the α III domain of the Δ *nifB* MoFe protein, which involves the significant repositioning of a number of residues. Some of these residues belong to a stretch of polypeptide ranging from α 353 to α 364 which, upon repositioning by distances up to 20 Å, forms a loop at the entrance of the funnel. Among them are the positively charged Arg α 359 and Arg α 361, as well as the His α 362, which could serve as the first contact point for FeMoco during the insertion process. Moving into the funnel, the C α of His α 442 is relocated by a distance of approximately 5 Å and joins His α 274 and His α 451 to form a striking His triad (Fig. 1), which could help guide the negatively charged FeMoco to the destined location. The movement of His α 442 also leads to a switch in position of this residue with Trp α 444, which could help secure FeMoco at its final location. Additionally, Lys α 426 is shifted by roughly 5 Å, which could serve as another anchoring point for FeMoco during insertion. These observations have led to the proposed mechanism of FeMoco insertion, which involves the initial docking of FeMoco at the entrance loop of the funnel, the continued cruising of FeMoco along the funnel under the guidance of the His triad, the anchoring of the Mo/homocitrate end of FeMoco by His α 442 and Lys α 426 at the bottom of the funnel, the swapping of positions of Trp α 444 and His α 442 that results in the capture of the FeMoco, and finally the closing up of the funnel by the movement of the entrance loop.

To test the structurally-based hypothesis of FeMoco insertion, we have constructed a series of MoFe protein variants with site-directed mutations of the key residues for this process and subjected them to extensive biochemical, biophysical and spectroscopic analyses. Recently, we showed that substitutions of Trp α 444 for small residues, or, His α 362 for uncharged or

negatively charged residues drastically decreased the level of FeMoco accumulation [19,20]. In this study we examine the role of two His triad residues, His α 274 and His α 451, in the process of FeMoco insertion. Our data clearly indicate that mutations of His α 274 and/or His α 451 to small uncharged Ala result in dramatically reduced levels of FeMoco incorporation, indicating that these residues are important for FeMoco insertion and that they likely interact with FeMoco through transitory ligand formation and/or electrostatic attraction during the insertion process.

2. Materials and methods

2.1. Reagents

Unless noted otherwise, all chemicals and reagents were obtained from Fisher Scientific, Baxter Scientific, or Sigma.

2.2. Construction of *A. vinelandii* Variant Strains

Table 1 summarizes the *A. vinelandii* strains used in this study. From this point on, the MoFe protein is designated as Av1, whereas the Fe protein is referred to as Av2. The mutations of the Av1 proteins are indicated by the appropriate superscripts. Note that the Av2 purified from the same strain as the Av1 variant are given the same superscript; however, the mutation is located in Av1, not Av2. The wild-type strain AvYM13A^{wild-type} (expressing His-tagged Av1^{wild-type} and non tagged Av2^{wild-type}) was constructed as described elsewhere [19]. The variant strains AvYM14A ^{α H274A} (expressing His-tagged Av1 ^{α H274A} and non tagged Av2 ^{α H274A}), AvYM22A ^{α H451A} (expressing His-tagged Av1 ^{α H451A} and non tagged Av2 ^{α H451A}) and AvYM23A ^{α H274A/ α H451A} (expressing His-tagged Av1 ^{α H274A/ α H451A} and non tagged Av2 ^{α H274A/ α H451A}) were constructed as follows. First, plasmid pHR30 was constructed, which contained the chromosomal fragment of *nifD* and *nifK* genes of *A. vinelandii*. Then, two oligos were used to create desired site-directed mutations of the *nifD* gene carried on pHR30, following the procedure of the commercially available GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega, Madison, WI). The oligos used for mutations were (i) H274A, 5'-AAGCTTAACCTGGTTGCCTGCTACCGCTCGATG-3' and (ii) H451A, 5'-TATTCGGGGCCCTACGCCGGCTTCGATGGCTTC-3'. Oligos (i) and (ii) were either used alone to create single mutation or together to create double mutations. The resulting plasmids were pHR38 (α H274A), pHR39 (α H451A) and pHR40 (α H274A/ α H451A). Finally, pHR38, pHR39 and pHR40 were transformed into AvYM13A using a previously described method [21,22]. The resulting variant strains were AvYM14A ^{α H274A}, AvYM22A ^{α H451A} and AvYM23A ^{α H274A/ α H451A}, respectively, with site-directed mutations of *nifD* gene on the chromosomal DNA. Additionally, the *nifB* gene in AvYM14A ^{α H274A}, AvYM22A ^{α H451A} or AvYM23A ^{α H274A/ α H451A} was deleted as described elsewhere [21]. The resulting strains were AvYM24A ^{Δ nifB/ α H274A} (expressing His-tagged Av1 ^{Δ nifB/ α H274A}), AvYM32A ^{Δ nifB/ α H451A} (expressing His-tagged Av1 ^{Δ nifB/ α H451A}) and AvYM33A ^{Δ nifB/ α H274A/ α H451A} (expressing His-tagged Av1 ^{Δ nifB/ α H274A/ α H451A}), respectively, with the deletion of *nifB* gene in addition to the site-directed mutations of *nifD* gene on the chromosomal DNA. The site-directed mutations of *nifD* were subsequently confirmed by DNA sequencing.

2.3. Cell Growth and Protein Purification

All *A. vinelandii* strains were grown in 180 L batches in a 200 L New Brunswick fermentor (New Brunswick Scientific, Edison, NJ) in Burke's minimal medium supplemented with 2 mM ammonium acetate. The growth rate was measured by cell density at 436 nm using Spectronic 20 Genesys (Spectronic Instruments, Westbury, NY). After the consumption of ammonia, cells were de-repressed for 3 h followed by harvesting using a flow through centrifugal harvester (Cepa, Lahr/Schwarzwald, Germany). The cell paste was washed with

50 mM Tris-HCl (pH 8.0). Published methods were used for the purification of all Av2 proteins [23] and His-tagged Av1 proteins [11,21]. FeMoco was isolated as described elsewhere [24].

2.4. EPR Spectroscopic Studies

All EPR samples were prepared in a Vacuum Atmospheres dry box (Hawthorne, CA) with an oxygen level of less than 4 ppm. Unless noted otherwise, all samples were in 25 mM Tris-HCl (pH 8.0), 10% glycerol and 2 mM Na₂S₂O₄. Av1 protein samples were oxidized by incubation with excess indigo disulfonate (IDS) for 30 min. Subsequently, IDS was removed by a single passage over an anion-exchange column as described elsewhere [25]. All perpendicular and parallel mode EPR spectra were recorded using a Bruker ESP 300 E_z spectrophotometer (Bruker, Billerica, MA), interfaced with an Oxford Instruments ESR-9002 liquid helium continuous flow cryostat (Oxford Instruments, Oxon, U.K.). Unless noted otherwise, all spectra were recorded at 10 K using a microwave power of 50 mW, a gain of 5×10^4 , a modulation frequency of 100 kHz, and a modulation amplitude of 5 G. The microwave frequencies of 9.62 and 9.39 GHz were used for the perpendicular (10 scans) and parallel (20 scans) mode EPR spectra, respectively. Spin quantitation of EPR signals was carried out as described in detail earlier [21].

2.5. Activity, Metal, FeMoco Insertion, FeMoco Maturation and Iron Chelation Assays

All nitrogenase activity assays and FeMoco insertion assays were carried out as described previously [21,24]. The products H₂ and C₂H₄ were analyzed as published elsewhere [26]. Ammonium was determined by a high performance liquid chromatography fluorescence method [27]. FeMoco maturation assays were carried out as published earlier [11]. Molybdenum [28] and iron [29] were determined as published elsewhere. The iron chelation assays were performed as described elsewhere [30,31] using an Fe protein concentration of 0.2 mg/ml.

2.6. Protein Stability Experiments

Two approaches were used to determine the stability of the purified Av1 proteins: (i) heat treatment and (ii) prolonged storage at room temperature. A total amount of 100 mg purified Av1 [in 25 mM Tris-HCl (pH 8.0), 10% glycerol, 250 mM NaCl and 2 mM Na₂S₂O₄] was incubated in a crimped anaerobic vial (volume, 8.7 ml; gas atmosphere, 100% Ar) at 56°C for 30 s (i) or stored at room temperature for 8 h (ii). Precipitated protein was subsequently removed in both cases by centrifugation at 10,000 rpm for 10 min (Biofuge *fresco*, Heraeus, Germany) in a Vacuum Atmospheres dry box. Concentrations of Av1 proteins were subsequently determined by Bio-Rad protein assays (Bio-Rad Laboratories, Hercules, CA). Enzymatic activities were determined as described above.

3. Results

His α 274 and His α 451 are highly conserved (both ~80%) among the currently known MoFe protein primary sequences². In Av1 ^{Δ nifB}, the snapshot of Av1 prior to the final step of FeMoco insertion, His α 274 and His α 451 are joined by His α 442 to form a remarkable His triad half way down the insertion funnel (Fig. 1) [18]. It has been hypothesized that such a histidine-rich core provides a midway docking point for FeMoco along its insertion pathway, during which process the Mo end of FeMoco makes the first contact with one of its eventual ligands, His α 442 [18]. Thus, the His triad is crucial for the on-target cruising of FeMoco toward its destination and disruption of the histidine enrichment at this particular point may prevent the

²These numbers are based on a sequence comparison of known nitrogenase MoFe proteins (SWISS-PROT) using the program BLAST [32].

FeMoco from being properly inserted into its target location. Based on this theory, a series of *A. vinelandii* strains expressing Av1 protein variants with site-directed mutations at the His triad was constructed. To minimize the mutational effect that may result in the instability of the protein, the FeMoco ligand His α 442 was left untouched; whereas His α 274 and His α 451 were mutated, either singularly or simultaneously, to small uncharged Ala. These *A. vinelandii* variant strains (Table 1), designated AvYM14A ^{α H274A} (expressing Av1 ^{α H274A} and Av2 ^{α H274A}), AvYM22A ^{α H451A} (expressing Av1 ^{α H451A} and Av2 ^{α H451A}) and AvYM23A ^{α H274A/ α H451A} (expressing Av1 ^{α H274A/ α H451A} and Av2 ^{α H274A/ α H451A}), respectively, were used in studies described in sections 3.1, 3.2 and 3.3.1–3.3.4. Additionally, a series of *nifB*-deletion *A. vinelandii* strains expressing the respective FeMoco-deficient forms of the His triad Av1 protein variants were constructed. These variant strains (Table 1), designated AvYM24A ^{Δ nifB/ α H274A} (expressing Av1 ^{Δ nifB/ α H274A}), AvYM32A ^{Δ nifB/ α H451A} (expressing Av1 ^{Δ nifB/ α H451A}) and AvYM33A ^{Δ nifB/ α H274A/ α H451A} (expressing Av1 ^{Δ nifB/ α H274A/ α H451A}), respectively, were used in studies described in section 3.3.5.

3.1. The His Triad Variant Strains Grow Slower under N₂-fixing Conditions

Under N₂-fixing conditions³, the doubling time of AvYM13A^{wild-type} is approximately 4.6 h; while those of AvYM14A ^{α H274A}, AvYM22A ^{α H451A} and AvYM23A ^{α H274A/ α H451A} are 4.9 h, 5.3 h and 5.9 h, respectively, all of which are longer than that of AvYM13A^{wild-type}. Considering that cell growth under N₂-fixing conditions reflects the amount of active nitrogenase, these results indicate that mutations of the His triad residues to small uncharged Ala result in decreased nitrogenase activities. As expected, double mutations of His α 274 and His α 451 present a more serious problem to the protein than a single mutation of either His residue, consistent with a greater extent of disruption of the histidine-rich environment at this particular location when both His residues are replaced by Ala.

3.2. Av2 Proteins of the His Triad Variant Strains Are Fully Competent

Reduced growth rates of the His triad variant strains do not originate from altered expression and properties of Av2⁴. The Av2 proteins of AvYM14A ^{α H274A}, AvYM22A ^{α H451A} and AvYM23A ^{α H274A/ α H451A} are expressed at nearly the same level as Av2^{wild-type}, as shown by Western blot analysis under non-saturated conditions (Fig. 2A and 2B). In addition, an approximate amount of 400 mg non-tagged Av2 was purified from 200 g cells of AvYM14A ^{α H274A}, AvYM22A ^{α H451A} or AvYM23A ^{α H274A/ α H451A}, as was from AvYM13A^{wild-type} (data not shown)⁵, again confirming that Av2 expression is unperturbed in these variant strains. The monomer of Av2 ^{α H274A}, Av2 ^{α H451A} or Av2 ^{α H274A/ α H451A}, like that of Av2^{wild-type}, is ~30 kDa (Fig. 3A). The molecular masses of all of these Av2 proteins are ~60 kDa based on their elution profiles on gel filtration columns (data not shown), suggesting that they all have the same, homodimeric subunit composition as Av2^{wild-type}. Compared to Av2^{wild-type}, all Av2 proteins of the His triad variant strains have the same metal content of approximately 4 mol Fe/mol protein (Table 2) and display, in the dithionite-reduced state, the same characteristic $S = 1/2$ EPR signal of rhombic line shape in the $g = 2$ region (Fig. 4). These data suggest that, like Av2^{wild-type}, all of these Av2 proteins have, per dimer, one [4Fe-4S] cluster that can be reduced by dithionite to an oxidation state of +1. All Av2 proteins of the

³Only organisms capable of expressing active nitrogenase enzymes are able to grow under the N₂-fixing conditions, where no fixed or organic nitrogen (such as ammonia and urea) is present in the culture media.

⁴Although point mutations of Av1 have no direct effect on Av2, it has been well established that Av2 is involved in FeMoco biosynthesis [8]. Since this work deals with FeMoco insertion, it is crucial to establish the catalytic and biosynthetic proficiency of Av2 in these variant strains.

⁵Note that for protein purification, all strains were grown in the presence of a limited amount of ammonia, so that a certain amount of cell mass could be accumulated regardless of the strain's ability to fix nitrogen. After the consumption of ammonia, cells were de-repressed for 3 h, during which process the nitrogenase protein (active or inactive) was expressed. Under these conditions, a consistent yield of ~200 g cells per 180 L cell growth batch could be obtained for the wild-type and variant strains of *A. vinelandii*.

His triad variant strains show the same substrate reducing activities as Av2^{wild-type}, with regard to C₂H₄ formation under C₂H₂/Ar, H₂ formation under Ar, NH₃ formation under N₂, or H₂ formation under N₂ (data not shown), suggesting that they are as efficient as Av2^{wild-type} in nitrogenase catalysis. In addition, all of them have the same capacity as Av2^{wild-type} in FeMoco maturation assay (data not shown), indicating that they are as functional as Av2^{wild-type} in FeMoco assembly.

3.3. Av1 Proteins of the His Triad Variant Strains Are Specifically Defective in FeMoco Insertion

Mutations at the His triad do not interfere with the accumulation of a certain amount of Av1 in cells, nor do they affect the structural integrity of Av1. The Av1 proteins of the His triad variant strains are expressed at approximately the same amount as Av1^{wild-type}, as evidenced by Western blot analysis under non-saturated conditions (Fig. 2A and 2C). Additionally, nearly 600 mg His-tagged Av1 was purified from 200 g cells of AvYM14A^{αH274A}, AvYM22A^{αH451A} or AvYM23A^{αH274A/αH451A}, as was from AvYM13A^{wild-type} (data not shown)⁵, further suggesting that Av1 expression is unaffected in these variant strains. Moreover, like Av1^{wild-type}, the Av1 proteins of all these variant strains are composed of α (~56 kDa)- and β (~59 kDa)-subunits (Fig. 3B) and have the same, α₂β₂-tetrameric molecular mass of ~230 kDa based on their elution profiles on gel filtration columns (data not shown).

Nevertheless, disruptions of the His triad specifically affect FeMoco insertion, as shown by combined metal, activity, EPR, stability and insertion studies of the His triad Av1 variant proteins (see below).

3.3.1. Metal Determination—The His triad Av1 variants contain reduced amounts of Fe and Mo (Table 2). The Mo contents of Av1^{αH274A}, Av1^{αH451A} and Av1^{αH274A/αH451A} are 61%, 50% and 44%, respectively, of that of Av1^{wild-type} (Table 2). Given that the Mo level reflects the FeMoco content, these results provide the first indication that mutations of the His triad residues result in decreased accumulation of FeMoco in the Av1 protein.

3.3.2. Activity Assay—Consistent with the reduced Mo content, the His triad Av1 variants have reduced substrate reducing activities. Compared to Av1^{wild-type}, the His triad Av1 variants show decreased activities of C₂H₄ formation under C₂H₂/Ar, H₂ formation under Ar, and NH₃ formation under N₂ (Table 3). Further, the extent of decrease in activities, in the ascending order of Av1^{αH274A} < Av1^{αH451A} < Av1^{αH274A/αH451A}, is consistent with the extent of decrease in growth rates of the strains expressing these proteins (see above). However, all three Av1 variants display unusually high capacities of H₂ formation under N₂—111%, 77% and 75%, respectively, of that of Av1^{wild-type}, which do not correlate proportionally with their capacities of NH₃ formation under the same conditions—51%, 46% and 36%, respectively, of that of Av1^{wild-type} (Table 3). This discrepancy indicates a shift toward H₂ evolution during the concomitant formation of NH₃ and H₂ under N₂, a situation where neither NH₃ nor H₂ formation alone reflects the overall activity of the reaction. To obtain an accurate measure of the substrate reducing activity under N₂, the total electron flux in the reaction was calculated on the basis of the electron pairs appearing in both products (NH₃ and H₂) and compared to those in the formation of C₂H₄ under C₂H₂/Ar and the formation of H₂ under Ar (Table 4). These calculations reveal that, each Av1 variant shows consistently reduced activities with regard to all substrates, which are 59%–74% of Av1^{wild-type} activity for Av1^{αH274A}, 53%–61% of Av1^{wild-type} activity for Av1^{αH451A}, and 40%–48% of Av1^{wild-type} activity for Av1^{αH274A/αH451A} (Table 4). On average, Av1^{αH274A}, Av1^{αH451A} and Av1^{αH274A/αH451A} have 67%, 56% and 43%, respectively, of the activity of Av1^{wild-type} (Table 4). These activities correlate well with the Mo contents of the respective Av1 variant proteins (Table 2), strongly

suggesting that the diminished activities of the His triad variants originate specifically from decreased levels of FeMoco in the Av1 proteins.

3.3.3. EPR Analysis—EPR analyses provide further evidence that disturbance of the His triad has significant impact on the FeMoco center while hardly affecting the P-cluster at all. In the IDS-oxidized state, Av1^{αH274A} (Fig. 5A, trace 2), Av1^{αH451A} (Fig. 5A, trace 3) and Av1^{αH274A/αH451A} (Fig. 5A, trace 4) show the P-cluster (P²⁺ state) specific, $g = 11.8$, parallel mode EPR signals [34,35], which integrate to 97%, 97% and 95%, respectively, of that of Av1^{wild-type} (Fig. 5A, trace 1). This observation suggests the presence of largely intact P-clusters in these Av1 variant proteins⁶. In the dithionite-reduced state, however, although the FeMoco-specific, $S = 3/2$, perpendicular mode EPR signal [1] of Av1^{wild-type} (Fig. 5B, trace 1) can be observed in the spectra of Av1^{αH274A} (Fig. 5B, trace 2), Av1^{αH451A} (Fig. 5B, trace 3) and Av1^{αH274A/αH451A} (Fig. 5B, trace 4), the signal intensities in all three cases are significantly reduced, indicating a decreased FeMoco content in these Av1 variants. Indeed, the yields of FeMocos isolated from Av1^{αH274A} (designated FeMoco^{αH274A}), Av1^{αH451A} (designated FeMoco^{αH451A}) and Av1^{αH274A/αH451A} (designated FeMoco^{αH274A/αH451A}) are 52%, 44% and 39%, respectively, of that from the same amount of Av1^{wild-type} (designated FeMoco^{wild-type}) (Table 5). These numbers are consistent with the Mo contents (Table 2) and the average activities (Table 4) of these variant Av1 proteins, once again suggesting that the His triad mutations specifically affect the accumulation of FeMoco in Av1.

Although the FeMocos isolated from all three His triad Av1 variants are qualitatively indistinguishable from FeMoco^{wild-type} with regard to spectroscopic features (data not shown) and substrate reducing activities (Table 5), within the proteins—particularly those carrying the mutation of His α274 to Ala, they show EPR features that are altered from that of the wild-type (Fig. 5B). Compared to the $S = 3/2$ signal of Av1^{wild-type} ($g = 4.32, 3.65, 2.01$), those of Av1^{αH274A} ($g = 4.47, 3.45, 1.98$) and Av1^{αH274A/αH451A} ($g = 4.44, 3.45, 1.98$) are broader in line shape (Fig. 5B, traces 1, 2 and 4). In addition, Av1^{αH274A} shows extra features at $g = 5.93, 5.26$ and 4.65 (Fig. 5B, trace 2); whereas Av1^{αH274A/αH451A} displays additional features at $g = 5.93, 5.26$ and 4.67 (Fig. 5B, trace 4). Av1^{αH451A} (Fig. 5B, trace 3), on the other hand, exhibits the same $S = 3/2$ signal as Av1^{wild-type} (Fig. 5B, trace 1), albeit at much reduced intensity.

The perturbed $S = 3/2$ signal in Av1^{αH274A} or Av1^{αH274A/αH451A} indicates a change in the immediate protein environment surrounding the FeMoco, rather than the presence of other EPR active species in the protein. Despite their altered shapes, the $S = 3/2$ signals of Av1^{αH274A} (Fig. 6A and B, trace 2) and Av1^{αH274A/αH451A} (Fig. 6A and B, trace 4) show the same temperature- and power-dependency as the normally shaped signals of Av1^{wild-type} (Fig. 6A and B, trace 1) and Av1^{αH451A} (Fig. 6A and B, trace 3). Upon variation of temperature or power, the intensities of the extra EPR features in the spectra of Av1^{αH274A} ($g = 5.93, 5.26$ and 4.65) and Av1^{αH274A/αH451A} ($g = 5.93, 5.26$ and 4.67) remain proportional to those of the major features of the $S = 3/2$ signal, reaching a maximum at 6 K (Fig. 6A) or 50 mW (Fig. 6B). Thus, these additional features are associated, exclusively, with the same cluster that gives rise to the main features of the $S = 3/2$ signal, *i.e.*, the FeMoco center in Av1. Given that His α274 is positioned closer to Cys α275 (one of the two FeMoco ligands) than His α451 (Fig. 1), it is not surprising that mutation of His α274 has a more severe impact on the FeMoco-specific $S = 3/2$ signal than that of His α451.

⁶In the dithionite-reduced state, Av1^{αH274A}, Av1^{αH451A} and Av1^{αH274A/αH451A} show additional, minor $S = 1/2$ signals that integrate to roughly 0.04, 0.05 and 0.10 spin per protein, respectively (Fig. 5B, traces 2–4). This unique signal has been previously associated with a P-cluster analog, which is composed of two [4Fe-4S]-like fragments [10]. It is likely, therefore, that mutations of the His triad residues near the FeMoco binding site somehow affect the P-cluster site and force a small portion of the substructural units of the [8Fe-7S] P-clusters slightly apart. Nevertheless, this portion of P-cluster species is negligible since the P-cluster (P²⁺ state) specific, parallel-mode EPR signal of all three Av1 variants integrate to nearly 100% of that of the wild type protein (Fig. 5A)

3.3.4. Protein Stability Test—Reduced accumulation of FeMoco in the His triad Av1 variants does not result from non-specific denaturation or incorrect folding of the proteins. Based on structural calculations by program *MUMBO* [36], mutations of His $\alpha 274$ and/or His $\alpha 451$ to Ala present no steric problems for the overall stability of Av1 (data not shown). This prediction is corroborated by the observation that Av1 $\alpha H274A$, Av1 $\alpha H451A$ and Av1 $\alpha H274A/\alpha H451A$ exhibit the same stability as Av1^{wild-type} upon heat treatment or prolonged storage at room temperature (data not shown). Furthermore, like Av1^{wild-type}, Av1 $\alpha H274A$, Av1 $\alpha H451A$ or Av1 $\alpha H274A/\alpha H451A$ can form a similarly stable complex with Av2^{wild-type} in the presence of MgATP (Fig. 7). All these results indicate that mutations at the His triad do not alter the stability of the protein, and that the observed decrease in FeMoco accumulation is indeed a specific outcome of altering the His triad residues that are important for FeMoco insertion.

3.3.5. FeMoco Insertion Experiment—To further assess the effect of the His triad mutations, Av1 $\alpha H274A$, Av1 $\alpha H451A$ and Av1 $\alpha H274A/\alpha H451A$ expressed in *nifB*-deletion background—namely, Av1 $\Delta nifB/\alpha H274A$, Av1 $\Delta nifB/\alpha H451A$ and Av1 $\Delta nifB/\alpha H274A/\alpha H451A$, respectively (Table 1), were subjected to *in vitro* FeMoco insertion experiments. Like the Av1 $\Delta nifB$ (containing the “wild-type” His triad), Av1 $\Delta nifB/\alpha H274A$, Av1 $\Delta nifB/\alpha H451A$ and Av1 $\Delta nifB/\alpha H274A/\alpha H451A$ are $\alpha_2\beta_2$ tetramers that are FeMoco-deficient yet P-cluster-replete (data not shown). However, while Av1 $\Delta nifB$ can be fully reconstituted by isolated FeMoco, Av1 $\Delta nifB/\alpha H274A$, Av1 $\Delta nifB/\alpha H451A$ and Av1 $\Delta nifB/\alpha H274A/\alpha H451A$ can only be reconstituted to reduced levels (Fig. 8). Furthermore, reconstitution reactions of Av1 $\Delta nifB/\alpha H274A$, Av1 $\Delta nifB/\alpha H451A$ and Av1 $\Delta nifB/\alpha H274A/\alpha H451A$ occur at significantly slower rates than Av1 $\Delta nifB$, especially during the initial phase (Fig. 8, inset). The levels of FeMoco incorporation under *in vitro* conditions—in the ascending order of Av1 $\Delta nifB/\alpha H274A/\alpha H451A$ < Av1 $\Delta nifB/\alpha H451A$ < Av1 $\Delta nifB/\alpha H274A$ < Av1 $\Delta nifB$, reflect the amounts of FeMoco accumulation under *in vivo* conditions—in the increasing sequence of Av1 $\alpha H274A/\alpha H451A$ < Av1 $\alpha H451A$ < Av1 $\alpha H274A$ < Av1^{wild-type}, convincingly making the case that the His triad variants are specifically defective in FeMoco insertion.

4. Discussion

Histidine is capable of coordinating metals in the cluster-containing proteins, and there is no exception for that in the case of nitrogenase. In Av1^{wild-type}, His $\alpha 442$ serves as one of the two protein ligands for FeMoco by ligating the Mo at the Mo/homocitrate end of the cofactor. In Av1 $\Delta nifB$, His $\alpha 442$ is grouped with two other histidines, His $\alpha 274$ and His $\alpha 451$, in forming a histidine-enriched cluster midway down the insertion funnel. It is very likely that, along with His $\alpha 442$, His $\alpha 274$ and His $\alpha 451$ provide an intermediate docking point for the FeMoco by transiently ligating to the Mo atom of the FeMoco. Meanwhile, His ($pK_a \approx 6$) is positively charged, at least partially, under physiological conditions ($pH \approx 6-7$). Thus, in addition to coordinating the FeMoco through the Mo atom, the His triad could also provide a positively charged milieu that further attracts the FeMoco through its negatively charged homocitrate entity. Either way, our data clearly indicate that the histidine enrichment at this position is important for FeMoco incorporation. It is plausible that the temporary docking of FeMoco at the His triad allows His $\alpha 442$ to establish contact with the Mo atom of FeMoco and subsequently guide the FeMoco to its target location in a swinging action that covers a distance of around 5 Å (Fig. 1, arrow).

In addition to playing a pivotal role in the final step of FeMoco assembly, the His triad residues are, apparently, also closely involved in the process of substrate reduction. In contrast to the mutations of Trp $\alpha 444$ or His $\alpha 362$ (other key residues for FeMoco insertion) to Ala, which lead to proportionally reduced production of NH_3 and H_2 [19,20], mutations of His $\alpha 274$ and/or His $\alpha 451$ to Ala cause a shift of N_2 reduction toward H_2 production (or proton reduction),

indicating a re-distribution of available electrons in favor of H₂ evolution (Table 3). Interestingly, early x-ray structural analysis of Av1^{wild-type} has led to the hypothesis that His α274 and His α451 are potentially involved in the shuffling of protons to the active site (FeMoco), a process that is indispensable for N₂ reduction [37]. Since understanding the concurrent production of H₂ during N₂ reduction is central to elucidating the overall reaction mechanism of nitrogenase catalysis, the His triad residues could serve as ideal targets for future mechanistic studies of nitrogenase in this respect.

In summary, we show that His α274 and His α451 of Av1, part of a His triad, are specifically involved in FeMoco insertion. Mutations of His α274 and/or His α451 to small uncharged Ala result in significantly reduced levels of FeMoco accumulation, indicating that the His triad is important for FeMoco incorporation, potentially serving as the intermediate docking point for FeMoco through transitory ligand coordination and/or electrostatic interaction. Future studies will focus on structural analysis of the His triad Av1 variants so that the precise functions of these important residues in nitrogenase assembly and catalysis can be defined.

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Abbreviations

FeMoco	iron-molybdenum cofactor
IDS	indigo disulfonate
Av1	MoFe protein
Av2	Fe protein
<i>nif</i>	nitrogen fixation genes

Fig. 1.

Overlaid protein environment of Av1^{wild-type} and Av1^{ΔnifB} in the vicinity of the FeMoco binding site. Parts of the C_α backbones of Av1^{wild-type} and Av1^{ΔnifB} are shown and colored as follows: Av1^{wild-type} in green and Av1^{ΔnifB} in light grey. FeMoco is shown in ball-and-stick presentation and colored as follows: oxygen in red, carbon in dark grey, molybdenum in orange, sulfur in yellow, iron in magenta. Note that, from this angle, only a small portion of the molybdenum atom is visible, while the central atom X (of unknown origin) is invisible. The side chains of His α274, His α451, His α442 and Cys α275 are shown in line presentation and colored as follows: His α274, His α451, His α442 of Av1^{wild-type} in green and blue, His α274, His α451, His α442 of Av1^{ΔnifB} in light grey and blue, Cys α275 of Av1^{wild-type} in green and yellow, and Cys α275 of Av1^{ΔnifB} in light grey and yellow. His α442 and Cys α275 are the two protein ligands that are covalently attached to FeMoco in Av1^{wild-type}. In Av1^{ΔnifB}, the position of Cys α275 remains largely unchanged, whereas His α442 is re-positioned by a distance of approximately 5 Å and joins His α451 and His α274 in the formation of a striking His triad, which presumably serves to guide the FeMoco to its final location. The distances between the His triad residues, measured by the distances between their respective C2 atoms of the imidazole rings (the carbon atom between the two ring nitrogen atoms) are: (i) 3.62 Å between His α451 and His α274; (ii) 3.60 Å between His α274 and His α442; and (iii) 3.66 Å between His α442 and His α451. Additionally, His α442 can potentially form a hydrogen bond with the nearby Asp α234.

Fig. 2.

Coomassie stained SDS-PAGE (A) and Western blots using Av2-specific (B) and Av1-specific (C) antibodies against cell-free crude extracts of *A. vinelandii* strains. (A) Lane 1, protein standard, 15 μ g; lane 2, AvYM13A^{wild-type} crude extract, 20 μ g; lane 3, AvYM14A ^{α H274A} crude extract, 20 μ g; lane 4, AvYM22A ^{α H451A} crude extract, 20 μ g; lane 5, AvYM23A ^{α H274A/ α H451A} crude extract, 20 μ g; lane 6, purified Av1^{wild-type}, 3.5 μ g; lane 7, purified Av2^{wild-type}, 3.5 μ g. (B) Lane 1, AvYM13A^{wild-type} crude extract, 20 μ g; lane 2, AvYM14A ^{α H274A} crude extract, 20 μ g; lane 3, AvYM22A ^{α H451A} crude extract, 20 μ g; lane 4, AvYM23A ^{α H274A/ α H451A} crude extract, 20 μ g; lane 5, purified Av2^{wild-type}, 3.5 μ g. (C) Lane 1, AvYM13A^{wild-type} crude extract, 20 μ g; lane 2, AvYM14A ^{α H274A} crude extract, 20 μ g; lane 3, AvYM22A ^{α H451A} crude extract, 20 μ g; lane 4, AvYM23A ^{α H274A/ α H451A} crude extract, 20 μ g; lane 5, purified Av1^{wild-type}, 3.5 μ g. Note that Western blot analyses were performed under non-saturated conditions.

Fig. 3.

Coomassie stained SDS-PAGE of purified Av2 (A) and Av1 (B) proteins. (A) 10–20% gradient SDS-PAGE of Av2 proteins: *lane 1*, protein standard, 15 μ g; *lane 2*, Av2^{wild-type}, 4 μ g; *lane 3*, Av2 ^{α H274A}, 4 μ g; *lane 4*, Av2 ^{α H451A}, 4 μ g; *lane 5*, Av2 ^{α H274A/ α H451A}, 4 μ g. The molecular masses of all purified Av2 proteins in this study appeared to be identical based on their elution profiles on gel filtration Sephacryl S-200 HR columns (Amersham Biosciences). (B) 4–15% gradient SDS-PAGE of Av1 proteins: *lane 1*, protein standard, 15 μ g; *lane 2*, Av1^{wild-type}, 5 μ g; *lane 3*, Av1 ^{α H274A}, 5 μ g; *lane 4*, Av1 ^{α H451A}, 5 μ g; *lane 5*, Av1 ^{α H274A/ α H451A}, 5 μ g. The molecular masses of all purified Av1 proteins in this study appeared to be identical based on their elution profiles on gel filtration Sephacryl S-200 HR columns (Amersham Biosciences).

Fig. 4. Perpendicular mode EPR spectra of dithionite-reduced Av2^{wild-type} (1), Av2^{αH274A} (2), Av2^{αH451A} (3) and Av2^{αH274A/αH451A} (4). EPR samples (20 mg/ml) were prepared and measured as described in “*Materials and methods*”. All Av2 proteins show the characteristic $S = 1/2$ signal of the $[4\text{Fe-4S}]^{1+}$ cluster at nearly the same intensity.

Fig. 5. (A) Parallel mode EPR spectra of IDS-oxidized Av1^{wild-type} (1), Av1^{αH274A} (2), Av1^{αH451A} (3) and Av1^{αH274A/αH451A} (4). EPR samples (20 mg/ml) were prepared and measured as described in “Materials and methods”. The P-cluster specific (P^{2+} state), $g = 11.8$, parallel mode EPR signals of Av1^{αH274A}, Av1^{αH451A} and Av1^{αH274A/αH451A} integrate to 97%, 97% and 95% of that of Av1^{wild-type}, respectively. (B) Perpendicular mode EPR spectra of dithionite-reduced Av1^{wild-type} (1), Av1^{αH274A} (2), Av1^{αH451A} (3) and Av1^{αH274A/αH451A} (4). EPR samples (20 mg/ml) were prepared and measured as described in “Materials and methods”. The FeMoco-specific, $S = 3/2$ EPR signals of Av1^{αH274A}, Av1^{αH451A} and Av1^{αH274A/αH451A} integrate to 44%, 58% and 29% of that of Av1^{wild-type}, respectively. The

$S = 1/2$ EPR signals in the $g \approx 2$ region of the spectra of $\text{Av1}^{\alpha\text{H274A}}$, $\text{Av1}^{\alpha\text{H451A}}$ and $\text{Av1}^{\alpha\text{H274A}/\alpha\text{H451A}}$ integrate to approximately 0.04, 0.05 and 0.10 spin per protein, respectively. The g values are indicated.

Fig. 6. Temperature- (A) and power- (B) dependency of the perpendicular mode EPR signals exhibited by dithionite-reduced Av1^{wild-type} (1), Av1^{αH274A} (2), Av1^{αH451A} (3) and Av1^{αH274A/αH451A} (4). All spectra were measured at a protein concentration of 20 mg/ml between 6 and 20 K at 50 mW (A) or between 5 and 50 mW at 10 K (B) as described in “Materials and methods”. The g values are indicated.

Fig. 7.

Protection of chelation of iron from the $[4\text{Fe-4S}]^{1+}$ cluster of $\text{Av2}^{\text{wild-type}}$ by complex formation of $\text{Av2}^{\text{wild-type}}$ with $\text{Av1}^{\text{wild-type}}$ (2), $\text{Av1}^{\alpha\text{H274A}}$ (3), $\text{Av1}^{\alpha\text{H451A}}$ (4) and $\text{Av1}^{\alpha\text{H274A}/\alpha\text{H451A}}$ (5). Formation of the complex between the iron chelator bathophenanthroline disulfonate and the iron from the $[4\text{Fe-4S}]^{1+}$ cluster of the $\text{Av2}^{\text{wild-type}}$ was measured at 535 nm in the presence of MgATP alone (1), MgATP plus Av1 proteins (2–5), or MgADP alone (6). Final concentrations of 0.4 mM MgCl_2 and 0.2 mM of either ADP or ATP were used. The concentrations of Av1 and Av2 proteins were 0.35 and 0.2 mg/ml, respectively. Curves obtained in the presence of MgATP and Av1 proteins were fitted to single exponential equations over a period of 20 s, giving the following observed rate constants: 0.0014 s^{-1} (1), 0.0008 s^{-1} (2), 0.0009 s^{-1} (3), 0.0008 s^{-1} (4), 0.0009 s^{-1} (5) and 0 s^{-1} (6). These studies show that each His triad Av1 variant protein forms a complex with $\text{Av2}^{\text{wild-type}}$ that is similarly stable compared to that formed between $\text{Av1}^{\text{wild-type}}$ and $\text{Av2}^{\text{wild-type}}$.

Fig. 8. Time-dependent FeMoco insertion into Av1 Δ nifB (●), Av1 Δ nifB/ α H274A (○), Av1 Δ nifB/ α H451A (▼) and Av1 Δ nifB/ α H274A/ α H451A (▽). FeMoco insertion was carried out as described in “Materials and methods”. The inset shows the first minute of the insertion, giving observed rates at 63% (Av1 Δ nifB/ α H274A), 54% (Av1 Δ nifB/ α H451A) and 36% (Av1 Δ nifB/ α H274A/ α H451A), respectively, of that of Av1 Δ nifB (set at 100%).

Table 1

Designations of *A. vinelandii* strains, proteins and FeMocos in this work

Strain	Strain designation	Genotype	Designation of MoFe protein ^{a,b}	Designation of Fe protein ^{a,b}	Designation of isolated FeMoco
YM13A	<i>Av</i> YM13A wild-type	His-tagged MoFe protein	<i>Av</i> 1 wild-type	<i>Av</i> 2 wild-type	FeMoco ^{wild-type}
YM14A	<i>Av</i> YM14A α H274A	His-tagged MoFe protein with site-directed α H274A mutation	<i>Av</i> 1 α H274A	<i>Av</i> 2 α H274A	FeMoco α H274A
YM22A	<i>Av</i> YM22A α H451A	His-tagged MoFe protein with site-directed α H451A mutation	<i>Av</i> 1 α H451A	<i>Av</i> 2 α H451	FeMoco α H451A
YM23A	<i>Av</i> YM23A α H274A/ α H451A	His-tagged MoFe protein with site-directed α H274A/ α H451A mutation	<i>Av</i> 1 α H274A/ α H451A	<i>Av</i> 2 α H274A/ α H451A	FeMoco α H274A/ α H451A
YM24A	<i>Av</i> YM24A Δ <i>nifB</i> / α H274A	His-tagged MoFe protein with site-directed α H274A mutation, deletion of <i>nifB</i> gene	<i>Av</i> 1 Δ <i>nifB</i> / α H274A	-- <i>c</i>	-- <i>d</i>
YM32A	<i>Av</i> YM32A Δ <i>nifB</i> / α H451A	His-tagged MoFe protein with site-directed α H451A mutation, deletion of <i>nifB</i> gene	<i>Av</i> 1 Δ <i>nifB</i> / α H451A	-- <i>c</i>	-- <i>d</i>
YM33A	<i>Av</i> YM33A Δ <i>nifB</i> / α H274A/ α H451A	His-tagged MoFe protein with site-directed α H274A/ α H451A mutation, deletion of <i>nifB</i> gene	<i>Av</i> 1 Δ <i>nifB</i> / α H274A/ α H451A	-- <i>c</i>	-- <i>d</i>
DJ1165	<i>Av</i> DJ1165	His-tagged MoFe protein, deletion of <i>nifH</i> gene	<i>Av</i> 1 Δ <i>nifH</i>	-- <i>e</i>	-- <i>d</i>
DJ1143	<i>Av</i> DJ1143	His-tagged MoFe protein, deletion of <i>nifB</i> gene	<i>Av</i> 1 Δ <i>nifB</i>	-- <i>c</i>	-- <i>d</i>

^aThe component proteins of the Mo-nitrogenase are designated by the initials of the organism from which they are isolated and the Arabic numeral of the component. For example, the MoFe protein and Fe protein of the Mo-nitrogenase of *A. vinelandii* are designated *Av*1 and *Av*2, respectively.

^bNote that, although *Av*1 and *Av*2 from the same variant strain have the same superscript, the site-directed mutations are located in *Av*1, not *Av*2.

^cNot used in this study.

^dNote that these *Av*1 proteins are FeMoco-deficient, hence no isolation of FeMoco in these cases.

^eNote that the *Av*2-encoding *nifH* gene is deleted in *Av*DJ1165, hence no expression of *Av*2 in this case.

Table 2

Metal contents of purified Av1 and Av2 proteins

Protein	Metal			
	Fe		Mo ^a	
	<i>mol metal/mol protein</i>	%	<i>mol metal/mol protein</i>	%
Av1 ^{wild-type}	29.3 ± 0.4	100	1.8 ± 0.2	100
Av1 ^{aH274A}	27.5 ± 2.6	94	1.1 ± 0.2	61
Av1 ^{aH451A}	25.8 ± 3.1	88	0.9 ± 0.1	50
Av1 ^{aH274A/aH451A}	24.2 ± 1.3	83	0.8 ± 0.1	44
Av2 ^{wild-type}	3.8 ± 0.4	100	ND ^b	
Av2 ^{aH274A}	4.0 ± 0.4	105	ND ^b	
Av2 ^{aH451A}	3.9 ± 0.3	103	ND ^b	
Av2 ^{aH274A/aH451A}	3.6 ± 0.6	95	ND ^b	

^aNote that the amount of Mo reflects the content of FeMoco.^bND = not determined

Table 3

Activities of purified Av1 proteins

Protein	Activities							
	C ₂ H ₄ -formation under C ₂ H ₂ /Ar		H ₂ -formation under Ar		NH ₃ -formation under N ₂		H ₂ -formation under N ₂	
	<i>nmol/min/mg</i> <i>protein</i>	%	<i>nmol/min/mg</i> <i>protein</i>	%	<i>nmol/min/mg</i> <i>protein</i>	%	<i>nmol/min/mg</i> <i>protein</i>	%
Av1 ^{wild-type}	2283 ± 73	100	2568 ± 117	100	1063 ± 133	100	678 ± 21	100
Av1 ^{αH274A}	1337 ± 31	59	1891 ± 20	74	538 ± 110	51	751 ± 64	111
Av1 ^{αH451A}	1207 ± 180	53	1554 ± 90	61	492 ± 34	46	524 ± 44	77
Av1 ^{αH274A/αH451A}	912 ± 44	40	1039 ± 89	40	384 ± 103	36	509 ± 51	75

^aNote that, although measured separately, NH₃ and H₂ are concomitantly produced under N₂ in the following reaction: N₂ + 8H⁺ + 8e⁻ → 2NH₃ + H₂.

Table 4

Activities based on electron pairs appearing in products

Protein	Condition ^a	Electron pair appearing in product ^b			Sum of activity ^{c,d}	Average activity ^e
		C ₂ H ₄	H ₂	NH ₃		
Av1 wild-type	C ₂ H ₂ /Ar	2283 ± 73	--	--	2283 ± 73 (100)	]
	Ar	--	2568 ± 117	--	2568 ± 117 (100)	{
	N ₂	--	678 ± 21	1595 ± 200	2273 ± 221 (100)	]
Av1 ^{Δt274A}	C ₂ H ₂ /Ar	1337 ± 31	--	--	1337 ± 31 (59)	]
	Ar	--	1891 ± 20	--	1891 ± 20 (74)	{
	N ₂	--	751 ± 64	807 ± 165	1558 ± 229 (69)	]
Av1 ^{Δt451A}	C ₂ H ₂ /Ar	1207 ± 180	--	--	1207 ± 180 (53)	]
	Ar	--	1554 ± 90	--	1554 ± 90 (61)	{
	N ₂	--	524 ± 44	738 ± 51	1262 ± 95 (56)	]
Av1 ^{Δt274A/Δt451A}	C ₂ H ₂ /Ar	912 ± 44	--	--	912 ± 44 (40)	]
	Ar	--	1039 ± 89	--	1039 ± 89 (40)	{
	N ₂	--	509 ± 51	576 ± 155	1085 ± 206 (48)	]

^aProducts formed under the conditions listed in the table are (i) C₂H₄ (under C₂H₂/Ar), (ii) H₂ (under Ar) and (iii) NH₃ and H₂ (under N₂). The reactions for the formation of these products are (i) C₂H₂ + 2H⁺ + 2e⁻ → C₂H₄, (ii) 2H⁺ + 2e⁻ → H₂ and (iii) N₂ + 8H⁺ + 8e⁻ → 2NH₃ + H₂.

^bActivity expressed as nmol electron pair appearing in product/min/mg protein, calculated by multiplying activity in nmol product/min/mg protein by the number of electron pairs appearing in each product, namely, 1, 1 and 1.5, respectively, for C₂H₄, H₂ and NH₃ (also see footnote a, above). This approach has been used previously to calculate the specific activities of nitrogenase reactions that involve the concomitant formation of multiple products [33].

^cThe sum of activity under each condition, calculated by adding activities, expressed in nmol electron pair/min/mg protein, of all products concurrently formed in the same reaction. Thus calculated activity represents the total electron flux per reaction, which is unaffected by the potential uneven distribution of electrons among the concurrently formed products and hence an accurate measure for activities in reactions involving multiple products.

^dPercentages of activities of Av1 variants in comparison to that of Av1 wild-type are given in parentheses.

^eThe average activities of each Av1 protein, calculated by adding the individual activities of C₂H₄ formation under C₂H₂/Ar, H₂ formation under Ar and NH₃/H₂ formation under N₂ (all of which expressed in total electron flux per reaction) and dividing the total by 3.

Table 5

Activities and yields of isolated FeMocos

FeMoco	Activities ^a						Yield ^b	
	C ₂ H ₄ -formation under C ₂ H ₂ /Ar	H ₂ -formation under Ar	NH ₃ -formation under N ₂	H ₂ -formation under N ₂	NH ₃ -formation under N ₂	H ₂ -formation under N ₂	FeMoco isolation	
	nmol/min/mg protein	%	nmol/min/mg protein	%	nmol/min/mg protein	nmol/min/mg protein	nmol Mo	%
FeMoco ^{wild-type}	813 ± 145	100	1016 ± 31	100	417 ± 45	217 ± 27	1430 ± 67	100
FeMoco ^{ΔH274A}	731 ± 7	90	968 ± 68	95	409 ± 32	180 ± 12	744 ± 19	52
FeMoco ^{ΔH451A}	858 ± 44	106	954 ± 52	94	425 ± 23	247 ± 27	630 ± 31	44
FeMoco ^{ΔH274A/ΔH451A}	882 ± 20	108	1167 ± 33	115	427 ± 61	202 ± 23	562 ± 16	39

^aThe activities were determined on the basis of the capabilities of isolated FeMocos to reconstitute Av1^{ΔnifB}, a FeMoco-depleted yet P-cluster-replete form of Av1 that can be converted to an active, holo-protein upon FeMoco insertion [18].

^bThe yield of FeMoco was determined by Mo analysis of FeMoco isolated from the same amount (300 mg) of Av1 protein.