Molecular Insights into Nitrogenase FeMoco Insertion

TRP-444 OF MoFe PROTEIN α-SUBUNIT LOCKS FeMoco IN ITS BINDING SITE

Yilin Hu, Aaron W. Fay, Benedikt Schmid, Beshoie Makar, and Markus W. Ribbe

From the Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92697-3900 and the Department of Biotechnology, Friedrich-Alexander-Universität Erlangen-Nürnberg, 91052 Erlangen, Germany

Biosynthesis of the FeMo cofactor (FeMoco) of nitrogenase MoFe protein is arguably one of the most complex processes in metalloprotein biochemistry. Here we investigate the role of a MoFe protein residue (Trp-α444) in the final step of FeMoco assembly, which involves the insertion of FeMoco into its binding site. Mutations of this aromatic residue to small uncharged ones result in significantly decreased levels of FeMoco insertion/retention and drastically reduced activities of MoFe proteins, suggesting that Trp-α444 may lock the FeMoco tightly in its binding site through the sterically restricting effect of its bulky, aromatic side chain. Additionally, these mutations cause partial conversion of the P-cluster to a more open conformation, indicating a potential connection between FeMoco insertion and P-cluster assembly. Our results provide some of the initial molecular insights into the FeMoco insertion process and, moreover, have useful implications for the overall scheme of nitrogenase assembly.

Nitrogenase is a multicomponent metallo-enzyme that catalyzes the reduction of atmospheric dinitrogen to bioavailable ammonia (for recent reviews see Refs. 1–8). The best studied, Mo-containing nitrogenase of Azotobacter vinelandii is composed of two proteins, the Fe protein and the MoFe protein. The homodimeric Fe protein (Av2) has one ATP binding site per subunit and a single [4Fe-4S] cluster bridged between the subunits; whereas the αβ tetramer MoFe protein (Av1) contains, in each αβ subunit pair, two complex metal clusters. One, designated the P-cluster, is an [8Fe-7S] cluster (9) that is located at the αβ interface and coordinated by six Cys ligands. The other, designated FeMoco, is a [Mo-7Fe-9S-X]3 cluster that can be subsequently inserted into its final location within the MoFe protein (10). These metal clusters are essential for nitrogenase reactivity, mediating the ATP-driven, interprotein electron transfer from the [4Fe-4S] cluster of the Fe protein to the P-cluster of the MoFe protein and finally to the FeMoco where substrate reduction takes place.

Biosynthesis of P-cluster and FeMoco has attracted considerable attention, because these clusters are biologically unique and chemically unprecedented. Both P-cluster and FeMoco are composed of smaller substructures: the P-cluster consists of two [4Fe-4S] subclusters sharing a μ₆-sulfide (9); and the FeMoco comprises [Mo-3Fe-3S] and [4Fe-3S] subcubanes sharing three μ₆-sulfides and a central μ₆-atom (10). Nonetheless, while the P-cluster is likely assembled through fusion of its substructural [4Fe-4S] units in its target location (11–13), FeMoco is first assembled on a scaffold NifEN protein and then inserted into its binding site in the MoFe protein (14–17). Biosynthesis of FeMoco presumably starts with the production of an Fe/S core by NifB (encoded by nifB), which is then transferred to, and further processed on the NifEN complex (14–19). Previously, we identified a molybdenum-free, NifEN-bound FeMoco precursor that bears a striking resemblance to the Fe/S core of the mature FeMoco (15). Most recently, we showed that, upon MgATP hydrolysis, Fe protein inserts molybdenum and homocitrate into the FeMoco precursor while it is still bound to NifEN, resulting in the formation of a fully complemented cluster that can be subsequently inserted into its final location in the MoFe protein (16, 17). We also established that the transfer of the cluster from NifEN to MoFe protein likely occurs through direct protein-protein interactions (16, 17). These findings not only provide important insights into the biosynthesis and biomimetic chemical synthesis of FeMoco, they may also bear useful implications for the biosynthetic mechanism of other complex metal-containing clusters (20–22).

Though progress has been made toward understanding the assembly of FeMoco prior to its insertion into the FeMoco binding site, little is known about the final step of FeMoco incorporation into its target location within the MoFe protein. Our current knowledge in this regard is largely based upon the crystal structure of a ΔnifB MoFe protein (Av1ΔnifB) from a nifB-deletion strain of A. vinelandii (23). Consistent with the hypothesis that nifB encodes for a protein that is involved in the biosynthesis of FeMoco, Av1ΔnifB is FeMoco-deficient, yet it contains normal P-clusters (23). In vitro, Av1ΔnifB can be reconstituted, without additional factors, into an active holoprotein by isolated FeMoco (23, 24). Given that the FeMoco binding site is fully buried at ~10 Å below the surface of the wild-type MoFe protein (Av1wild-type), this observation indicates that Av1ΔnifB undergoes significant conformational rearrangements relative to its holo counterpart, a process that renders its FeMoco site open and allows the subsequent insertion of isolated FeMoco (23). Indeed, when compared with Av1wild-type, the αII domain of Av1ΔnifB has some major structural changes in that nearly all β-strands are shorter toward their C termini while α-helices A,
C, and D are shorter toward their N termini. These conformational alterations create a positively charged insertion funnel in Av1\(^{\Delta w.1}\), notably missing in its holo counterpart, that presumably steers the negatively charged FeMoco all the way down the funnel into its final location (23). A closer look at the FeMoco binding sites of Av1\(^{\text{wild-type}}\) and Av1\(^{\Delta w.1}\) (Fig. 1) reveals that, in the case of Av1\(^{\Delta w.1}\), the C\(_{\alpha}\) of His-\(\varepsilon 442\) shifts ~5 Å during the rearrangement of \(\alpha III\) domain and joins two other residues, His-\(\varepsilon 274\) and His-\(\varepsilon 451\), in the formation of a striking His triad (23). Coupled to this rearrangement, residues His-\(\varepsilon 442\) and Trp-\(\varepsilon 444\) switch their relative positions (23). Additionally, substantial changes take place in non-liganding residues as well; particularly, the stretch from \(\alpha 355\) to \(\alpha 359\), with Gly-\(\delta 356\), Gly-\(\varepsilon 357\), and Arg-\(\varepsilon 359\) that normally form hydrogen bonds to FeMoco sulfurs in holo Av1 (23). These residues are likely the key players in the process of FeMoco insertion and, therefore, serve as ideal targets for us to tackle the mechanism of the final step of FeMoco assembly on a molecular basis.

In the current study we examine the role of Trp-\(\varepsilon 444\) of Av1 in FeMoco insertion by combined mutational, biochemical, and spectroscopic approaches. Our data show that substitution of this large aromatic residue for small uncharged ones results in a drastically decreased level of FeMoco insertion/retention, thus suggesting that the sterically prohibiting Trp-\(\varepsilon 444\) may function in the FeMoco insertion process by tightly locking the FeMoco into its binding site.

EXPERIMENTAL PROCEDURES

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

Construction of Variant A. vinelandii Strains—Table 1 summarizes the A. vinelandii strains used in this study. The wild-type strain AvYM13A\(^{\text{wild-type}}\) (expressing His-tagged Av1\(^{\text{wild-type}}\) and non-tagged Av2\(^{\text{wild-type}}\) was constructed as described elsewhere (24). The variant strains AvYM18A\(^{W444A-nifD}\) (expressing His-tagged Av1\(^{W444A-nifD}\) and non-tagged Av2\(^{W444A-nifD}\)), AvYM19A\(^{W444F-nifD}\) (expressing His-tagged Av1\(^{W444F-nifD}\) and non-tagged Av2\(^{W444F-nifD}\)), AvYM20A\(^{W444G-nifD}\) (expressing His-tagged Av1\(^{W444G-nifD}\) and non-tagged Av2\(^{W444G-nifD}\)) and AvYM21A\(^{W444Y-nifD}\) (expressing His-tagged Av1\(^{W444Y-nifD}\) and non-tagged Av2\(^{W444Y-nifD}\)) were constructed as follows. First, plasmid pH30 was constructed, which contained the chromosomal fragment of nifD and nifK genes of A. vinelandii. Then, a series of oligos was used to create desired site-directed mutations of the nifD gene carried on pH30, following the procedure of the commercial GeneEditor in vitro Site-directed Mutagenesis System (Promega, Madison, WI). The oligos used for mutations were (i) W444A, 5’-CGTCAATGCAC-TCCGCCGATTATTCGGGCCC-3’; (ii) W444F, 5’-CGT-CAAATGACCTCTTCCGATTATTCGGGCCC-3’; (iii) W444G, 5’-CTCAATGACCTCTTCCGATTATTCGGGCCC-3’; and (iv) W444Y, 5’-CTCAATGACCTCTTCCGATTATTCGGGCCC-3’.

The resulting plasmids were pH31 (W444A-nifD), pH32 (W444F-nifD), pH33 (W444G-nifD), and pH34 (W444Y-nifD). Finally, pH31, pH32, pH33, and pH34 were transformed into AvYM13A using a previously described method (27, 28), resulting in variant strains AvYM18A\(^{W444A-nifD}\), AvYM19A\(^{W444F-nifD}\), AvYM20A\(^{W444G-nifD}\), and AvYM21A\(^{W444Y-nifD}\).
and AvYM21A<sup>W444Y</sup> with site-directed mutations of nifD gene on the chromosomal DNA.

**Cell Growth and Protein Purification**—All *A. vinelandii* strains were grown in 180-liter batches in a 200-liter 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 a Spectronic 20 Genesys (Spectronic Instruments, Westbury, NY). After the consumption of ammonia, the 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 (29) and His-tagged Av1 proteins (12, 28).

**EPR Spectroscopy**—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 (30). All perpendicular and parallel mode EPR spectra were recorded using a Bruker ESP 300 E<sub>x</sub> spectrophotometer (Bruker, Billerica, MA), interfaced with an Oxford Instruments ESR-9002 liquid helium continuous flow cryostat (Oxford Instruments, Oxon, UK). All spectra were recorded at 10 K using a microwave power of 50 milliwatt, a gain of 5 × 10<sup>4</sup>, a modulation frequency of 100 kHz, and a modulation amplitude of 5 Gauss. 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 (28).

**Activity Assays and Metal Analysis**—All nitrogenase activity assays were carried out as described previously (31). The products H<sub>2</sub> and C<sub>2</sub>H<sub>4</sub> were analyzed as published elsewhere (32). Ammonium was determined by a high performance liquid chromatography fluorescence method (33). FeMoco maturation assays were carried out as published earlier (14). Molybdenum (34) and iron (35) were determined as published elsewhere.

**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 of 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 12 h. A negligible amount of cell growth was observed in the case of (i) or (ii). Strains were grown in 10-liter batches in a 12-liters New Brunswick fermentor on Burke’s minimal medium. In all cases inoculums were 200 ml, with an approximate absorbance at 436 nm of 1.6. Growth of all strains was monitored by measuring the absorbance at 436 nm. The doubling time for each strain was determined by cell growth in the exponential phase as follows: (trace 1) 5 h, (trace 2) 5 h, (trace 3) 5 h, and (trace 5) 12 h. A negligible amount of cell growth was observed in the case of (trace 5).

**RESULTS AND DISCUSSION**

Trp-α444 is a highly conserved (≈80%) residue among the currently known MoFe proteins from various organisms; the rest of them contain Tyr (≈20%) in place of Trp. The 100% natural

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**TABLE 1**

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

| Strain    | Strain designation | Genotype          | Designation of MoFe protein<sup>a,b</sup> | Designation of Fe protein<sup>a,b</sup> |
|-----------|--------------------|-------------------|------------------------------------------|----------------------------------------|
| YM13A     | AvYM13A<sup>wild-type</sup> | His-tagged MoFe protein | Av1<sup>野生型</sup> | Av2<sup>野生型</sup> |
| YM18A     | AvYM18A<sup>nifD</sup> | His-tagged MoFe protein with site-directed W444A<sup>nifD</sup> mutation | Av1<sup>W444A-nifD</sup> | Av2<sup>W444A-nifD</sup> |
| YM19A     | AvYM19A<sup>nifD</sup> | His-tagged MoFe protein with site-directed W444F<sup>nifD</sup> mutation | Av1<sup>W444F-nifD</sup> | Av2<sup>W444F-nifD</sup> |
| YM20A     | AvYM20A<sup>nifD</sup> | His-tagged MoFe protein with site-directed W444G<sup>nifD</sup> mutation | Av1<sup>W444G-nifD</sup> | Av2<sup>W444G-nifD</sup> |
| YM21A     | AvYM21A<sup>nifD</sup> | His-tagged MoFe protein with site-directed W444Y<sup>nifD</sup> mutation | Av1<sup>W444Y-nifD</sup> | Av2<sup>W444Y-nifD</sup> |
| DJ1165    | AvDJ1165 | His-tagged MoFe protein, deletion of nifB gene | Av1<sup>ΔnifB</sup> | Av2<sup>ΔnifB</sup> |
| DJ1143    | AvDJ1143 | His-tagged MoFe protein, deletion of nifR gene | Av1<sup>ΔnifR</sup> | Av2<sup>ΔnifR</sup> |

<sup>a</sup> The 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 Av1 and Av2, respectively.

<sup>b</sup> Note that although Av1 and Av2 from the same variant strain have the same superscript, the site-directed mutations are located in Av1, not Av2.

<sup>c</sup> Note that the Av2-encoding nifH gene is deleted in AvDJ1165, hence no expression of Av2 in this case.

<sup>d</sup> Av2 of AvDJ1143 is not used in this study.
occurrence of an aromatic residue at this particular site, and the observation of a swapping in positions between this residue (Trp-α444) and one of the FeMoco ligands (His-α442) in Av1 δnifH, have led to the hypothesis that a switch between the two residues is required for the large aromatic α444 residue to firmly pack the FeMoco into its final location (23).

Mutations of this aromatic residue to small uncharged ones, therefore, may result in reduced capacities of the resulting Av1 variants in locking FeMoco in its binding site. A series of A. vinelandii strains expressing His-tagged Av1 protein variants with site-directed mutations at α444 was constructed, with Trp-α444 replaced with Gly (G), Ala (A), Phe (F), or Tyr (Y). These strains (Table 1) are designated Av1YM18AW444G- (expressing Av1 W444G-nifD and Av2 W444A-nifD), Av1YM19AW444F- (expressing Av1 W444F-nifD and Av2 W444A-nifD), Av1YM20AW444F- (expressing Av1 W444F-nifD and Av2 W444G-nifD), and Av1YM21AW444Y- (expressing Av1 W444Y-nifD and Av2 W444A-nifD).

Under N2-fixing conditions, the doubling time of Av1YM19AW444F- or Av1YM20AW444F-, like that of Av2 wild-type, is ~30 kDa (Fig. 3A). The molecular masses of all these Av2 proteins are ~60 kDa based on their elution profiles on gel filtration Sephacryl S-200 HR columns (data not shown), indicating that they are all homodimers. Compared with their wild-type counterpart, all Av2 proteins of the α444 variant strains have approximately the same metal content of 4 mol Fe/mol protein (Table 2) and exhibit, in the dithionite-reduced state, the same characteristic absorption spectrum as Av2 wild-type (Fig. 3B). Given that cell growth under N2-fixing conditions is proportionately correlated with the amount of active nitrogenase, these observations indicate that a mutation of the aromatic Trp residue at α444 to a small uncharged residue (in the case of Av1YM18AW444G- or Av1YM20AW444F-) results in a significantly diminished enzymatic activity of nitrogenase; whereas nitrogenase reactivity is largely unaffected when the Trp-α444 is replaced by another aromatic residue (in the case of Av1YM19AW444F- or Av1YM21AW444Y-). Apparently, the replacement of Trp-α444 with Gly has a more dramatic effect than that with Ala, which is not surprising, considering that Gly is a smaller residue than Ala. Meanwhile, although the doubling time of Av1YM19AW444F- or Av1YM21AW444Y- is roughly the same as that of Av1 YM13A wild-type, compared with Av1YM13A wild-type, Av1YM21AW444Y-nifD reaches nearly the same cell density while Av1YM19AW444F-nifD achieves a slightly reduced cell mass, suggesting that the change of Trp-α444 to Phe (no natural occurrence) has a more significant impact on the enzyme activity than that to Tyr (20% more activity). This could be explained by the fact that the side chain of Phe is slightly smaller than that of the Tyr or Trp.

The decreased enzymatic activities do not originate from the Av2 proteins of these α444 variant strains. An approximate amount of 400 mg of non-tagged Av2 was purified from 200 g of cells of Av1YM19AW444F-nifD, Av1YM20AW444F-nifD, Av1YM18AW444G-nifD, or Av1YM19AW444G-nifD as was from Av1 YM13A wild-type (data not shown), suggesting that Av2 expression is unaffected in these variant strains. The monomer of Av2 W444F-nifD, Av2 W444G-nifD, or Av2 W444G-nifD, like that of Av2 wild-type, is ~30 kDa (Fig. 3A). The molecular masses of all these Av2 proteins are ~60 kDa based on their elution profiles on gel filtration Sephacryl S-200 high resolution columns (data not shown), indicating that they are all homodimers. Compared with their wild-type counterpart, all Av2 proteins of the α444 variant strains have approximately the same metal content of 4 mol Fe/mol protein (Table 2) and exhibit, in the dithionite-reduced state, the same characteristic absorption spectrum as Av2 wild-type (Fig. 3B). Given that cell growth under N2-fixing conditions is proportionately correlated with the amount of active nitrogenase, these observations indicate that a mutation of the aromatic Trp residue at α444 to a small uncharged residue (in the case of Av1YM18AW444G- or Av1YM20AW444F-) results in a significantly diminished enzymatic activity of nitrogenase; whereas nitrogenase reactivity is largely unaffected when the Trp-α444 is replaced by another aromatic residue (in the case of Av1YM19AW444F- or Av1YM21AW444Y-). Apparently, the replacement of Trp-α444 with Gly has a more dramatic effect than that with Ala, which is not surprising, considering that Gly is a smaller residue than Ala. Meanwhile, although the doubling time of Av1YM19AW444F- or Av1YM21AW444Y- is roughly the same as that of Av1 YM13A wild-type, compared with Av1YM13A wild-type, Av1YM21AW444Y-nifD reaches nearly the same cell density while Av1YM19AW444F-nifD achieves a slightly reduced cell mass, suggesting that the change of Trp-α444 to Phe (no natural occurrence) has a more significant impact on the enzyme activity than that to Tyr (20% more activity). This could be explained by the fact that the side chain of Phe is slightly smaller than that of the Tyr or Trp.

The decreased enzymatic activities do not originate from the Av2 proteins of these α444 variant strains. An approximate amount of 400 mg of non-tagged Av2 was purified from 200 g of cells of Av1YM19AW444F-nifD, Av1YM20AW444F-nifD, Av1YM18AW444G-nifD, or Av1YM19AW444G-nifD as was from Av1 YM13A wild-type (data not shown), suggesting that Av2 expression is unaffected in these variant strains. The monomer of Av2 W444F-nifD, Av2 W444G-nifD, or Av2 W444G-nifD, like that of Av2 wild-type, is ~30 kDa (Fig. 3A). The molecular masses of all these Av2 proteins are ~60 kDa based on their elution profiles on gel filtration Sephacryl S-200 high resolution columns (data not shown), indicating that they are all homodimers. Compared with their wild-type counterpart, all Av2 proteins of the α444 variant strains have approximately the same metal content of 4 mol Fe/mol protein (Table 2) and exhibit, in the dithionite-reduced state, the same characteristic absorption spectrum as Av2 wild-type (Fig. 3B).
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S = 1/2 EPR signal of rhombic line shape in the g = 2 region (Fig. 4). These data suggest that all Av2 proteins have a normal complement of [4Fe-4S] cluster and that the [4Fe-4S] cluster can be reduced by dithionite to an oxidation state of +1. All Av2 proteins show the same substrate reducing activities as Av2wild-type with regard to C2H4 formation under C2H2/Ar, H2 formation under Ar, NH3 formation under N2, or H2 formation under N2 (Table 3), suggesting that they are all fully proficient in their catalytic capacities. Additionally, these Av2 proteins all function as normally as the wild-type protein in the FeMoco maturation assay (Table 3), indicating that they are perfectly competent in FeMoco assembly, a second function that has been definitively assigned to this multitask component of nitrogenase recently (14, 16, 17).

Like the Av2 proteins, the Av1 proteins of the Trp-α444 variant strains are expressed at practically the same level as Av1wild-type, as evidenced by Western blot analysis of Av1-specific antibody against the crude extracts of these variant strains under non-saturated conditions (supplemental Fig. S1). In addition, ~600 mg of His-tagged Av1 was purified from 200 g of cells of AvYM21A W444V-nifD, AvYM20A W444F-nifD, AvYM18A W444A-nifD or AvYM19A W444G-nifD, as was from AvYM13A wild-type (data not shown), further supporting the notion that Av1 expression is unperturbed with point mutations at α444 of Av1. Moreover, like Av1wild-type, all these α444 Av1 variants are composed of α (~56 kDa)- and β (~59 kDa)-subunits (Fig. 3B) and have the same, α3β2-tetrameric molecular mass of ~230 kDa based on their elution profiles on gel filtration Sephacryl S-200 columns (data not shown). Given the presence of nearly the same amount of Av1 in the variant strains, the diminished nitrogenase activity, particularly in the case of AvYM18A W444A-nifD or AvYM20A W444G-nifD, must arise from a decrease in the activity of the variant Av1 protein. Indeed, compared with Av1wild-type, Av W444A-nifD (of AvYM18A W444A-nifD) has ~20% of activity in terms of C2H4 formation under C2H2/Ar, H2 formation under Ar, NH3 formation under N2, or H2 formation under N2; whereas Av1 W444G-nifD (of AvYM20A W444G-nifD) shows practically no activity at all (Table 4). On the other hand, Av1 W444V-nifD (of AvYM21A W444V-nifD) has nearly 100% of the activity of Av1wild-type; while Av1 W444F-nifD (of AvYM19A W444F-nifD) shows a moderately reduced activity that ranges between 81 and 92% of that of Av1wild-type (Table 4). The activities of Av1 proteins are consistent with the growth rates of the respective variant strains expressing them (Fig. 2); more importantly, they correlate well with their respective metal contents (Table 2). The amount of iron or molybdenum that is present in Av1 decreases as follows: Av1 W444V-nifD > W444A-nifD > W444F-nifD > W444Y-nifD > W444G-nifD.

TABLE 3
Activities of purified Av2 proteins

| Protein | C2H4 formation under C2H2/Ar | H2 formation under Ar | NH3 formation under N2 | H2 formation under N2 |
|---------|-------------------------------|----------------------|-----------------------|----------------------|
| (a) Substrate reduction | nmol/min/mg protein | % | nmol/min/mg protein | % | nmol/min/mg protein | % | nmol/min/mg protein | % |
| Av2 W444V-nifD | 2091 ± 18 | 100 | 2130 ± 107 | 100 | 889 ± 57 | 100 | 636 ± 18 | 100 |
| Av2 W444A-nifD | 2348 ± 215 | 112 | 2210 ± 104 | 104 | 920 ± 45 | 103 | 682 ± 52 | 107 |
| Av2 W444F-nifD | 2092 ± 113 | 100 | 2031 ± 28 | 95 | 904 ± 79 | 102 | 761 ± 75 | 120 |
| Av2 W444G-nifD | 2164 ± 139 | 103 | 2149 ± 67 | 101 | 898 ± 29 | 101 | 704 ± 115 | 111 |
| Av2 W444F-nifD | 2190 ± 58 | 105 | 2386 ± 184 | 112 | 927 ± 88 | 104 | 748 ± 68 | 118 |

(b) FeMoco maturation assay

| Protein | C2H4 formation under C2H2/Ar | H2 formation under Ar | NH3 formation under N2 | H2 formation under N2 |
|---------|-------------------------------|----------------------|-----------------------|----------------------|
| Av2 W444V-nifD | 301 ± 38 | 100 | 332 ± 23 | 100 | 148 ± 15 | 100 | 58 ± 2 | 100 |
| Av2 W444A-nifD | 349 ± 37 | 116 | 311 ± 11 | 94 | 156 ± 23 | 105 | 66 ± 2 | 114 |
| Av2 W444F-nifD | 323 ± 43 | 107 | 353 ± 4 | 106 | 144 ± 9 | 97 | 62 ± 7 | 107 |
| Av2 W444G-nifD | 330 ± 9 | 110 | 361 ± 34 | 109 | 161 ± 19 | 109 | 56 ± 10 | 97 |
| Av2 W444F-nifD | 318 ± 42 | 106 | 318 ± 40 | 96 | 153 ± 25 | 103 | 56 ± 4 | 97 |
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### TABLE 4
Activities and EPR spin integration of purified Av1 proteins

| Protein          | C2H4 formation under C2H2/Ar | H2 formation under Ar | NH3 formation under N2 | H2 formation under N2 | Spin integration of S = 3/2 EPR signal |
|------------------|-----------------------------|----------------------|------------------------|------------------------|---------------------------------------|
| Av1wild-type     | 2096 ± 47                   | 100                  | 2211 ± 44              | 100                    | 989 ± 16                              | 555 ± 19                            |
| Av1W444Y-mbD     | 2066 ± 111                  | 99                   | 2069 ± 96              | 94                     | 1036 ± 81                             | 507 ± 15                            |
| Av1W444F-mbD     | 1906 ± 120                  | 91                   | 1795 ± 69              | 81                     | 906 ± 59                              | 505 ± 22                            |
| Av1W444A-mbD     | 364 ± 22                    | 17                   | 410 ± 23               | 19                     | 212 ± 13                              | 101 ± 4                             |

*In contrast to the P-cluster replete yet FeMoco-depleted Av1 wild-type, which can be fully activated upon addition of isolated FeMoco, none of the Av1 variant proteins listed in the table can be further activated upon FeMoco addition (data not shown).*

FIGURE 5. Perpendicular mode EPR spectra of dithionite-reduced Av1 wild-type (trace 1), Av1W444Y-mbD (trace 2), Av1W444F-mbD (trace 3), and Av1W444A-mbD (trace 4). EPR samples (20 mg/ml) were prepared and measured as described under Experimental Procedures. The FeMoco-specific, S = 3/2 EPR signals of Av1W444Y-mbD, Av1W444F-mbD, and Av1W444A-mbD integrate to 98, 80, 21, and 1% of that of Av1wild-type, respectively. The S = 1/2 EPR signals in the g ~ 2 region of the spectra of Av1W444Y-mbD and Av1W444A-mbD integrate to 0.08 and 0.1 spin per Av1 wild-type, respectively.

Av1W444F-mbD > Av1W444A-mbD > Av1W444Y-mbD, which agrees with an increase in the extent of Trp-α444 mutation regarding the size of the residue. In particular, the molybdenum content (Table 2), in the descending order of Av1W444Y-mbD (91%), Av1W444F-mbD (87%), Av1W444A-mbD (14%), and Av1W444G-mbD (3%), is remarkably consistent with the activity of the respective protein (Table 4), in the decreasing rank of Av1W444Y-mbD (91–105%), Av1W444F-mbD (81–92%), Av1W444A-mbD (18–21%), and Av1W444G-mbD (<1%). Because the molybdenum content correlates directly with the FeMoco content, these results provide compelling evidence that the reduced activities of Av1 proteins from α444 variant strains originate from decreased levels of FeMoco in the proteins. Furthermore, the substrate reducing activities of all substrates, namely, C2H4 formation under C2H2/Ar, H2 formation under Ar, NH3 formation under N2, and H2 formation under N2, are proportionally decreased (Table 4). This observation suggests that the FeMoco is correctly inserted in the Av1 variant protein and indicates, yet again, that the decreased activity of Av1 variant protein is caused by less FeMoco rather than the incorrectly positioned FeMoco that could change the activity profile of Av1.

EPR analyses of the Av1 variant proteins provide further support to our argument that a mutation of Trp-α444 to a small uncharged residue results in a decreased amount of FeMoco accumulation in Av1. In the dithionite-reduced state, Av1wild-type (Fig. 5, trace 1) exhibits a distinct, FeMoco-specific, S = 3/2 signal (1). This S = 3/2 signal is also observed in the case of Av1W444Y-mbD (Fig. 5, trace 2), Av1W444F-mbD (Fig. 5, trace 3), or Av1W444A-mbD (Fig. 5, trace 4), although spin integration data indicate that the intensities of the signals are 98, 80, and 21% of that of Av1wild-type for Av1W444Y-mbD, Av1W444F-mbD, and Av1W444A-mbD, respectively (Table 4). In the case of Av1W444G-mbD, this characteristic S = 3/2 signal is virtually non-existent, integrating to 1% of that of Av1wild-type (Fig. 5, trace 5). The spin integration data from these EPR experiments align well with those from the growth curves (Fig. 2), metal analyses (Table 2) and activity assays (Table 4), once again confirming the presence of less FeMoco in Av1 variant proteins with mutations from aromatic Trp-α444 to small uncharged residues like Ala and Gly. Thus, all results (summarized in supplemental Fig. S2) come together and corroborate with our theory that Trp-α444 is specifically involved in FeMoco insertion, that the large size and steric rigidity of Trp-α444 is crucial for locking FeMoco into its binding site, and that removal of this restraint compromises the ability of the Av1 to insert and/or retain FeMoco.

Interestingly, in the dithionite-reduced state, Av1W444A-mbD (Fig. 5, trace 4) and Av1W444G-mbD (Fig. 5, trace 5) show additional S = 1/2 signals that integrate to 0.08 and 0.1 spin per protein, respectively. This particular S = 1/2 signal has been previously assigned to a P-cluster analog comprising two [4Fe-4S]-like fragments, which may represent a physiologi-
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FIGURE 6. Parallel mode EPR spectra of IDS-oxidized Av1wild-type (trace 1), Av1W444Y-nifD (trace 2), Av1W444F-nifD (trace 3), Av1W444A-nifD (trace 4), and Av1W444G-nifD (trace 5). EPR samples (20 mg/ml) were prepared and measured as described under "Experimental Procedures." The P-cluster specific (P^2− state), g = 11.8, parallel mode EPR signals of Av1W444Y-nifD, Av1W444F-nifD, Av1W444A-nifD, and Av1W444G-nifD integrated to 106, 99, 92, and 93% of that of Av1wild-type, respectively.

Table 5 Comparison of the stability of purified Av1 proteins

| Av1          | Amount of proteina | Specific activityb |
|--------------|--------------------|-------------------|
|              | Before  | After  | Before | After  |
| Av1wild-type | 100     | 97     | 2096 ± 47 | 2100 ± 40 |
| Av1W444Y-nifD| 100     | 93     | 2011 ± 47 | 1951 ± 67 |
| Av1W444F-nifD| 100     | 95     | 1971 ± 20 | 2038 ± 56 |
| Av1W444A-nifD| 100     | 95     | 348 ± 5   | 332 ± 69  |
| Av1W444G-nifD| 100     | 96     | 10 ± 1    | 7 ± 1     |

a Protein in mg, determined before and after heat treatment or prolonged storage as described under "Experimental Procedures."
b Specific activity in nmol of C2H4 evolution/min/mg protein.

The FeMoco-deficient, P-cluster precursor-containing Av1^moifD was used as a negative control in these experiments, which showed 90% and 64% of degradation, respectively, upon heat treatment and prolonged storage.

Av1 protein samples were incubated at 56 °C for 30 seconds as described under "Experimental Procedures."

Av1 protein samples were stored at room temperature for 8 hours as described under "Experimental Procedures."

It is important to note that, the mutations at Trp-α444 do not affect the overall stability of the Av1 variant proteins in this study. Like Av1^wild-type variant proteins Av1W444Y-nifD, Av1W444F-nifD, Av1W444A-nifD, and Av1W444G-nifD all exhibit considerable stability upon heat treatment or prolonged storage at room temperature (Table 5). In addition, in the presence of MgATP, each of these α444 Av1 variants can form a complex with Av2^wild-type that is similarly stable compared with that formed between Av1^wild-type and Av2^wild-type (supplemental Fig. S3). Furthermore, structural predictions based on program MUMBO (40) show that the Trp-α444 mutations in this study present no steric problems that could lead to the instability of the Av1 protein (data not shown).

In summary, using a combined mutational/bio-chemical/spectroscopic approach, we show that the Trp-α444 of Av1 is specifically involved in the FeMoco insertion/retention. Mutations of this aromatic residue to small uncharged ones result in dramatically decreased levels of FeMoco insertion/retention and drastically reduced activities of the Av1 proteins, suggesting that Trp-α444 may lock the FeMoco tightly into its binding site through the sterically restricting effect of its bulky, aro-

cally relevant intermediate during P-cluster assembly (12, 13, 37). Consistent with this observation, in the IDS-oxidized state, Av1 variant proteins exhibit P-cluster specific (P^2− state), g = 11.8, parallel mode EPR signals (38, 39), which integrate to 106, 99, 92, and 93% of that of Av1^wild-type (Fig. 6, trace 1) for Av1W444Y-nifD (Fig. 6, trace 2), Av1W444F-nifD (Fig. 6, trace 3), Av1W444A-nifD (Fig. 6, trace 4) and Av1W444G-nifD (Fig. 6, trace 5), respectively. The S = 1/2 signal-associated analog of P-cluster, therefore, could account for the missing portion of P-cluster in Av1W444A-nifD or Av1W444G-nifD. Apparently, when Trp-α444, one of the protein residues in a stretch of polypeptide that reorients with FeMoco insertion, is mutated to small uncharged residues, the [8Fe-7S] P-cluster is converted, partially, to a more open conformation consisting of a pair of [4Fe-4S]-like clusters. It is likely that the mutation of Trp-α444 to Ala or Gly leads to a conformational rearrangement that in turn affects the P-cluster site and forces the substructural units of P-cluster apart. This observation, therefore, suggests a potential association between the insertion of FeMoco and the assembly of P-clus-

The protein-design program MUMBO (40) was used to predict the stability of Av1 variant proteins, where Trp-α444 was replaced by Tyr, Phe, Ala, or Gly, respectively. Initially, MUMBO was tested for its ability to find the correct side chain conformations of those residues located less than 10 Å away from Trp-α444 (with Trp-α444 included). The predicted side chain conformations were a close match to those found in the crystal structure (Protein Data Bank entry 1MN1) thus confirming the feasibility of the program for this particular protein (data not shown). Subsequently, the side chain conformations in the same 10 Å region of the Av1 variant proteins were calculated with MUMBO for each case, which again led to a close match to those of Av1^wild-type. The mutated side chains were oriented similarly to the Trp side chain and energetically even more favorable (data not shown). Based on this prediction, no steric problems that could lead to the instability of the protein were observed.
matic side chain. Additionally, these mutations cause partial conversion of the P-cluster to a more open conformation, indicating a potential connection between FeMoco insertion and P-cluster assembly. Our results provide some of the initial insights into the process of FeMoco insertion on the molecular basis. Future studies will focus on addressing the effects of other key residues involved in FeMoco insertion and investigating the link between the assembly processes of FeMoco and P-cluster, in hope of elucidating a more detailed mechanism of nitrogense biosynthesis.

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